



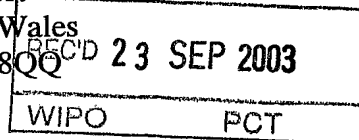
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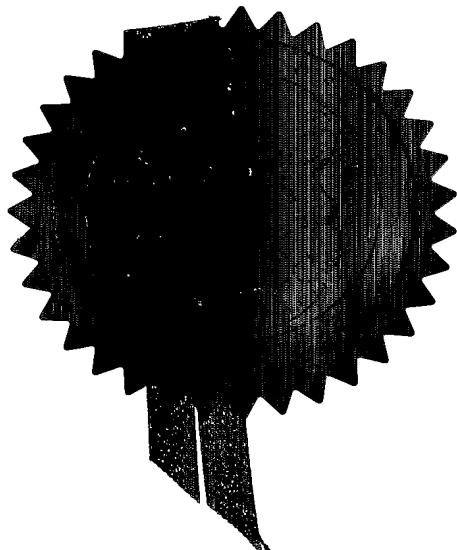


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23JUL02 E735288-1 D02866

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DELE / P13909GB

2. Patent application number

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23 JUL 2002

3. Full name, address and postcode of the or of each applicant (underline all surnames)

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Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

5677729001

4. Title of the invention

GENE AND POLYPEPTIDE SEQUENCES

5. Name of your agent (*if you have one*)

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ERIC POTTER CLARKSON

Date
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GENE AND POLYPEPTIDE SEQUENCES

The present invention relates to polypeptide and polynucleotide sequences for secreting proteins from host cells.

5

Numerous natural or artificial polypeptide signal sequences (also called secretion pre regions) have been used or developed for secreting desired peptides, polypeptides and proteins (these terms are used interchangeably from hereon in) from host cells. The signal sequence directs the nascent
10 protein towards the machinery of the cell that exports proteins from the cell into the surrounding medium or, in some cases, into the periplasmic space. The signal sequence is usually, although not necessarily, located at the N-terminus of the primary translation product and is generally, although not necessarily, cleaved off the desired protein during the secretion process, to
15 yield the "mature" protein.

In the case of some desired proteins the entity that is initially secreted, after the removal of the signal sequence, includes additional amino acids at its N-terminus called a "pro" sequence, the intermediate entity being called a
20 "pro-protein". These pro sequences may assist the final protein to fold and become functional, and are usually then cleaved off. In other instances, the pro region simply provides a cleavage site for an enzyme to cleave off the pre-pro region and is not known to have another function.

25 The pro sequence can be removed either during the secretion of the desired protein from the cell or after export from the cell into the surrounding medium or periplasmic space.

Polypeptide sequences which direct the secretion of proteins, whether they
30 resemble signal (i.e. pre) sequences or pre-pro secretion sequences, are

sometimes also referred to as leader sequences. The secretion of proteins is a dynamic process involving translation, translocation and post-translational processing, and one or more of these steps may not necessarily be completed before another is either initiated or completed.

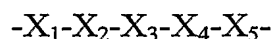
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For production of proteins in eukaryotic species such as the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, known leader sequences include those from the *S. cerevisiae* acid phosphatase protein (Pho5p) (see EP 366 400), the invertase protein (Suc2p) (see Smith *et al.* (1985) *Science*,
10 229, 1219-1224) and heat-shock protein-150 (Hsp150p) (see WO 95/33833). Additionally, leader sequences from the *S. cerevisiae* mating factor alpha-1 protein (MF α -1) and from the human lysozyme and human serum albumin (HSA) protein have been used, the latter having been used especially, although not exclusively, for secreting human albumin. WO
15 90/01063 discloses a fusion of the MF α -1 and HSA leader sequences, which advantageously reduces the production of a contaminating fragment of human albumin relative to the use of the MF α -1 leader sequence.

Unexpectedly, we have found that the yield of secreted protein can be
20 increased by the introduction of an amino acid sequence motif, preferably by modification of leader sequences. The modifications are effective whether made to the complete native albumin leader sequence, variants thereof, or to other leader sequences that employ the relevant part of the human albumin leader sequence, such as the fusion of MF α -1 and HSA
25 leader sequences as disclosed in WO 90/01063. In the latter case, if albumin is the protein secreted, the albumin thus produced retains the advantageous feature of reduced contaminating fragment, whilst still increasing the yield.

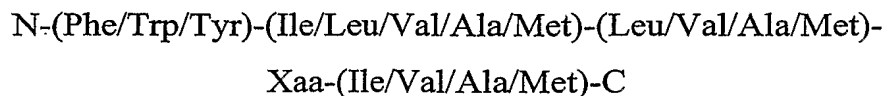
Although conservative modifications of the fused leader sequence of WO 90/01063 were disclosed in general terms in WO 90/01063 (for example, see page 8 of WO 90/01063), this resulted in a class of some 8×10^{12} polypeptides being defined. Polynucleotide coding sequences were set out for the exemplified leader sequence, according to the degeneracy of the genetic code. This also represents a large number of possibilities. There is no appreciation in WO 90/01063 that the specific class of modified leader sequences provided by the present invention would have advantageous properties for expression of secreted protein.

In a first aspect of the present invention there is provided a polypeptide comprising (i) a leader sequence, the leader sequence comprising (a) a secretion pre sequence and (b) the following motif:



where X_1 is phenylalanine, tryptophan, or tyrosine, X_2 is isoleucine, leucine, valine, alanine or methionine, X_3 is leucine, valine, alanine or methionine, X_4 is any amino acid and X_5 is isoleucine, valine, alanine or methionine; and (ii) a desired protein, heterologous to the leader sequence.

In other words, the polypeptide includes a sequence according to SEQ ID NO 1 –



SEQ ID No 1

(Ile/Val/Ala/Met)-C

SEQ ID No 5

5 In another preferred embodiment of the first aspect of the present invention,
X₄ is threonine. Thus another preferred polypeptide includes the sequence
of SEQ ID NO 29 -

N-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-Thr-
(Ile/Val/Ala/Met)-C

10

SEQ ID No 29

In another preferred embodiment of the first aspect of the present invention,
X₅ is isoleucine. Thus another preferred polypeptide includes the sequence
of SEQ ID NO 6 -

15

N-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-
Xaa-Ile-C

SEQ ID No 6

20

More preferably at least 2, even more preferably at least 3, yet more
preferably at least 4 of X₁, X₂, X₃, X₄ and X₅ are as defined in the preferred
embodiments above.

25 The motif may be inserted into the leader sequence (i.e. as an addition), or
can be included as a substitute for 1, 2, 3, 4, 5 or more contiguous amino
acids within the leader sequence.

In one preferred embodiment, the motif is included in the leader sequence
30 as a substitution for naturally occurring amino acids. In other words, the

amino acids of the motif are included in the place of five contiguous amino acids that were, or would have been, present in the leader sequence prior to its optimisation according to the present invention. The reader will appreciate that the phrase "naturally occurring" when used in this context, is not intended to limit the invention to the optimisation of naturally occurring leader sequences. On the contrary, this invention is also applicable to the optimisation of artificial leader sequences, such as the HSA/MF α -1 leader sequence fusion the optimisation of which is exemplified herein.

It is preferable that, where the motif is included in the leader sequence as a substitution then X_4 is the naturally occurring amino acid, or a variant thereof. In other words, preferably only X_1 , X_2 , X_3 and X_5 are substituted, whilst X_4 is maintained unchanged, or simply changed to a variant, preferably as a conservative substitution as defined below, of the natural amino acid at that position.

In a particularly preferred embodiment of the first aspect of the present invention, X_1 is phenylalanine, X_2 is isoleucine, X_3 is valine, X_4 is serine and X_5 is isoleucine. Thus in a particularly preferred embodiment of the first aspect of the invention, there is provided a polypeptide which includes the sequence of SEQ ID No 7 -

N-Phe-Ile-Val-Ser-Ile-C

25

SEQ ID No 7

In the above schemes, "N" and "C" denote the orientation of the polypeptide sequence, and are not intended to be limited in their interpretation to the actual termini; in other words, the polypeptide sequence may be joined (e.g. fused, conjugated or ligated), to one or more other

polypeptide sequences at either the N-, or C- ends, or most usually at both ends.

5 A polypeptide according to the first aspect of the invention comprises the sequence of a mature desired protein, heterologous to the leader sequence. A mature desired protein sequence is the primary amino acid sequence that will be present in the expression product following post-translational processing by the expression system in which the polypeptide of the invention is expressed. The desired protein is preferably suitable for
10 secretion from a cell in which the polypeptide of the invention is expressed.

The desired protein is heterologous to the leader sequence. In other words, the polypeptide of the first aspect of the present invention does not include naturally occurring proteins that have, in their leader sequences, the motif -
15 $X_1-X_2-X_3-X_4-X_5$ - as defined above. In a preferred embodiment, the polypeptide of the first aspect of the present invention does not include any naturally occurring protein that has the motif $-X_1-X_2-X_3-X_4-X_5$ - as defined above at any position. In this context, the term "naturally occurring" refers to proteins encoded by naturally occurring organisms that have not been
20 modified by recombinant technology, site-directed mutagenesis or equivalent artificial techniques that requires human intervention.

The desired protein may comprise any sequence, be it natural protein (including a zymogen), polypeptide or peptide, or a variant, or a fragment
25 (which may, for example, be a domain) of a natural protein, polypeptide or peptide; or a totally synthetic protein, polypeptide or peptide; or a single or multiple fusion of different proteins, polypeptides or peptides (natural or synthetic). Such proteins can be taken, but not exclusively, from the lists provided in WO 01/79258, WO 01/79271, WO 01/79442, WO 01/79443,
30 WO 01/79444 and WO 01/79480, or a variant or fragment thereof; the

disclosures of which are incorporated herein by reference. Although these patent applications present the list of proteins in the context of fusion partners for albumin, the present invention is not so limited and, for the purposes of the present invention, any of the proteins listed therein may be presented alone or as fusion partners for albumin, the Fc region of immunoglobulin, transferrin or any other protein as a desired polypeptide.

Preferred examples of a desired protein for expression by the present invention includes albumin, transferrin, lactoferrin, endostatin, angiostatin, collagens, immunoglobulins, Fab' fragments, F(ab')₂, ScAb, ScFv, interferons, IL10, IL11, IL2, interferon α species and sub-species, interferon β species and sub-species, interferon γ species and sub-species, IL1-receptor antagonist, EPO, TPO, prosaptide, cyanovirin-N, 5-helix, T20 peptide, T1249 peptide, HIV gp41, HIV gp120, fibrinogen, urokinase, prourokinase, tPA (tissue plasminogen activator), hirudin, platelet derived growth factor, parathyroid hormone, proinsulin, insulin, insulin-like growth factor, calcitonin, growth hormone, transforming growth factor β , tumour necrosis factor, G-CSF, GM-CSF, M-CSF, coagulation factors in both pre and active forms, including but not limited to plasminogen, fibrinogen, thrombin, pre-thrombin, pro-thrombin, von Willebrand's factor, α_1 -antitrypsin, plasminogen activators, Factor VII, Factor VIII, Factor IX, Factor X and Factor XIII, nerve growth factor, LACI (lipoprotein associated coagulation inhibitor, also known as tissue factor pathway inhibitor or extrinsic pathway inhibitor), platelet-derived endothelial cell growth factor (PD-ECGF), glucose oxidase, serum cholinesterase, aprotinin, amyloid precursor, inter-alpha trypsin inhibitor, antithrombin III, apo-lipoprotein species, Protein C, Protein S, a variant or fragment of any of the above.

A "variant", in the context of a desired protein, refers to a protein wherein at one or more positions there have been amino acid insertions, deletions, or

substitutions, either conservative or non-conservative, provided that such changes result in a protein whose basic properties, for example enzymatic activity or receptor binding (type of and specific activity), thermostability, activity in a certain pH-range (pH-stability) have not significantly been changed. "Significantly" in this context means that one skilled in the art would say that the properties of the variant may still be different but would not be unobvious over the ones of the original protein.

By "conservative substitutions" is intended combinations such as Val, Ile, Leu, Ala, Met; Asp, Glu; Asn, Gln; Ser, Thr, Gly, Ala; Lys, Arg, His; and Phe, Tyr, Trp. Preferred conservative substitutions include Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

A "variant" typically has at least 25%, at least 50%, at least 60% or at least 70%, preferably at least 80%, more preferably at least 90%, even more preferably at least 95%, yet more preferably at least 99%, most preferably at least 99.5% sequence identity to the polypeptide from which it is derived.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (Thompson *et al.*, (1994) *Nucleic Acids Res.*, 22(22), 4673-80). The parameters used may be as follows:

- Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

- Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.
- Scoring matrix: BLOSUM.

5 Such variants may be natural or made using the methods of protein engineering and site-directed mutagenesis as are well known in the art.

A "fragment", in the context of a desired proteins, refers to a protein wherein at one or more positions there have been deletions. Thus the fragment may
 10 comprise at most 5, 10, 20, 30, 40 or 50% of the complete sequence of the full mature polypeptide. Typically a fragment comprises up to 60%, more typically up to 70%, preferably up to 80%, more preferably up to 90%, even more preferably up to 95%, yet more preferably up to 99% of the complete sequence of the full desired protein. Particularly preferred fragments of a
 15 desired protein comprise one or more whole domains of the desired protein. For example, the desired protein may be albumin. Albumin has three domains. A particularly preferred fragment of albumin may contain one or two domains and will thus typically comprise at least 33% or at least 66% of the complete sequence of albumin.

20

Albumin and transferrin, or variants or fragments thereof, are particularly preferred as a desired protein, especially when they are of human origin, i.e. they have same sequence as that found in the naturally produced human protein.

25

The term "human albumin" is used herein to denote material which is indistinguishable from human serum albumin or which is a variant or fragment thereof. By "variant" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes
 30 do not substantially alter the oncotic, useful ligand-binding or immunogenic

properties of albumin. For example we include naturally-occurring polymorphic variants of human albumin or human albumin analogues disclosed in EP-A-322 094. Generally, variants or fragments of human albumin will have at least 10% (preferably at least 50%, 80%, 90% or 95%) of human serum albumin's ligand binding activity (for example bilirubin-binding) and at least 50% (preferably at least 80%, 90% or 95%) of human serum albumin's oncotic activity, weight for weight. Oncotic activity, also known as colloid osmotic pressure, of albumin, albumin variants or fragments of albumin may be determined by the method described by Hoefs, J.C. (1992) *Hepatology* 16:396-403. Bilirubin binding may be measured by fluorescence enhancement at 527 nm relative to HSA. Bilirubin (1.0mg) is dissolved in 50 μ L of 1M NaOH and diluted to 1.0mL with demineralised water. The bilirubin stock is diluted in 100mM Tris-HCl pH8.5, 1mM EDTA to give 0.6nmol of bilirubin mL⁻¹ in a fluorometer cuvette. Fluorescence is measured by excitation at 448nm and emission at 527nm (10nm slit widths) during titration with HSA over a range of HSA:bilirubin ratios from 0 to 5 mol:mol.

Similarly, the term "human transferrin" is used herein to denote material which is indistinguishable from transferrin derived from a human or which is a variant or fragment thereof. A "variant" includes insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the useful ligand-binding or immunogenic properties of transferrin. For example we include naturally-occurring polymorphic variants of human transferrin or human transferrin analogues. Generally, variants or fragments of human transferrin will have at least 50% (preferably at least 80%, 90% or 95%) of human transferrin's ligand binding activity (for example iron-binding), weight for weight. The iron binding activity of transferrin or a test sample can be determined spectrophotometrically by 470nm:280nm absorbance ratios for the proteins in their iron-free and fully iron-loaded states. Reagents should be iron-free

unless stated otherwise. Iron can be removed from transferrin or the test sample by dialysis against 0.1M citrate, 0.1M acetate, 10mM EDTA pH4.5. Protein should be at approximately 20mg/mL in 100mM HEPES, 10mM NaHCO₃ pH8.0. Measure the 470nm:280nm absorbance ratio of apo-transferrin (Calbiochem, CN Biosciences, Nottingham, UK) diluted in water so that absorbance at 280nm can be accurately determined spectrophotometrically (0% iron binding). Prepare 20mM iron-nitrilotriacetate (FeNTA) solution by dissolving 191mg nitrotriactic acid in 2mL 1M NaOH, then add 2mL 0.5M ferric chloride. Dilute to 50mL with deionised water. Fully load apo-transferrin with iron (100% iron binding) by adding a sufficient excess of freshly prepared 20mM FeNTA, then dialyse the holo-transferrin preparation completely against 100mM HEPES, 10mM NaHCO₃ pH8.0 to remove remaining FeNTA before measuring the absorbance ratio at 470nm:280nm. Repeat the procedure using test sample, which should initially be free from iron, and compare final ratios to the control.

Additionally, single or multiple heterologous fusions of any of the above; or single or multiple heterologous fusions to albumin, transferrin or immunoglobins or a variant or fragment of any of these may be used. Such fusions include albumin N-terminal fusions, albumin C-terminal fusions and co-N-terminal and C-terminal albumin fusions as exemplified by WO 01/79271, and transferrin N-terminal fusions, transferrin C-terminal fusions, and co-N-terminal and C-terminal transferrin fusions.

25

In a preferred embodiment, a polypeptide according to a first aspect of the invention comprises a secretion pre sequence that includes at least a part of the X₁-X₅ pentapeptide motif as defined above. In other words, the region of the leader sequence that acts to effect secretion of the mature desired polypeptide contains, 1, 2, 3, 4, or 5 of the amino acids of the X₁-X₅

30

pentapeptide motif. Where the secretion pre sequence region contains less than 5 amino acids of the X_1 - X_5 pentapeptide motif, those amino acids of the motif that are contained in the pre sequence are located at one of the borders of the pre sequence region, such that they are adjacent to the remaining amino acids of the X_1 - X_5 pentapeptide motif.

In a more preferred embodiment a polypeptide according to a first aspect of the present invention comprises a leader sequence characterised in that it includes a secretion pre sequence that includes the motif as defined above by the first aspect of the present invention. The leader sequence is usually, although not necessarily, located at the N-terminus of the primary translation product and is generally, although not necessarily, cleaved off the protein during the secretion process, to yield the mature "desired" protein.

A secretion leader sequence is usually, although not necessarily, an N-terminal sequence of amino acids that causes the polypeptide of which it forms part to be secreted from a host cell in which it is produced. Secretion is defined by the co-translational or post-translation translocation of a protein from the cytoplasmic compartment across a phospholipid bilayer, typically, but not exclusively the endoplasmic reticulum of eukaryotic organisms or the plasma membrane of prokaryotic organisms. The secreted protein may be retained within the confines of the cell (typically, but not exclusively, within the endoplasmic reticulum, Golgi apparatus, vacuole, lysosome or periplasmic space) or it may be secreted from the cell into the culture medium. A sequence acts as a secretion leader sequence if, in comparison to an equivalent polypeptide without the secretion pre sequence, it causes more of that polypeptide to be secreted from the host cell in which it is produced. Generally speaking, a polypeptide with a leader sequence will be secreted whereas a polypeptide without a leader sequence will not.

However, the present invention contemplates circumstances wherein different leader sequences will have different levels of efficiency. Thus a leader sequence may cause at least 10%, 20%, 30 or 40% or 50%, typically at least 60% or 70%, preferably at least 80%, more preferably at least 90%,
5 even more preferably at least 95%, yet more preferably at least 98%, most preferably at least 99% of the mature protein produced by the cell to be secreted from the cell. Secretion of a mature polypeptide from a cell can be determined, for example, by providing a host cell with appropriate DNA constructs and measuring the amount of the mature protein (for example,
10 human albumin) that is secreted, compared with any mature protein that is produced intracellularly.

A preferred secretion leader sequence will provide for the above mentioned levels of secretion when the host cell is a yeast cell (eg. *Saccharomyces*
15 *cerevisiae* or *Pichia pastoris*). Secretion of a mature polypeptide from a yeast host cell can be determined, for example, by methods such as those set out in the examples below.

Solubilised proteins from the cell biomass and secreted proteins in the culture
20 supernatant can be analysed by:

1. Gel permeation high pressure liquid chromatography.
2. Densitometry of SDS-PAGE
3. Rocket immunoelectrophoresis

The amount of the desired protein, secreted and intracellular, can be quantified
25 relative to a standard curve of the desired protein and normalised to the amount of biomass as known by those skilled in the art.

Usually it is preferable if the leader sequence is derived from the immature version of the mature protein to which it is, or is intended to be, attached.
30 Thus, for example, where the mature protein is albumin, it is preferred to

use sequences comprising the naturally occurring albumin secretion pre sequence, or pro sequence or pre-pro sequence. However, the leader sequence may alternatively be derived from a source other than that of the mature protein.

5

Thus in one preferred embodiment, the leader sequence of a polypeptide of the first aspect of the present invention comprises a secretion pre sequence derived from an albumin secretion pre sequence, or variant thereof.

10 A "variant" of an albumin pre sequence, as used above, refers to an albumin pre sequence wherein at one or more positions, other than at those defined by X_1 , X_2 , X_3 , X_4 or X_5 above, there have been amino acid insertions, deletions, or substitutions, either conservative (as described above) or non-conservative, provided that such changes still allow the peptide to act as a pre sequence.

15

Preferably, a "variant" of an albumin pre sequence has, other than the residues defined as X_1 - X_5 above, at least 2, at least 3 or at least 4, preferably at least 5, more preferably at least 6, even more preferably at least 7, yet more preferably at least 8, most preferably at least 9 identical amino acids to
20 a naturally occurring albumin pre sequence, most preferably the albumin pre sequence of Figure 1.

25

Even more preferably, where the secretion pre sequence is derived from an albumin secretion pre sequence, a polypeptide according to the first aspect of the present invention has X_1 , X_2 , X_3 , X_4 and X_5 at positions -20, -19, -18, -17 and -16, respectively, in place of the naturally occurring amino acids at those positions, wherein the numbering is such that the -1 residue is the C-terminal amino acid of the native albumin secretion pro sequence and where X_1 , X_2 , X_3 , X_4 and X_5 are amino acids as defined above

30

For example, when the above mentioned numbering is applied to the sequence of the human albumin secretion pre sequence (as disclosed, for example in WO 90/01063), the following is obtained:

5	N	-	Met	Lys	Trp	Val	Ser	Phe	Ile	Ser	Leu	Leu
			-24	-23	-22	-21	-20	-19	-18	-17	-16	-15
			Phe	Leu	Phe	Ser	Ser	Ala	Tyr	Ser	-	C
			-14	-13	-12	-11	-10	-9	-8	-7		

10

In a particularly preferred embodiment the secretion pre sequence used is derived from the sequence of the human albumin secretion pre sequence.

Thus, for example, the X₁-X₅ pentapeptide may be fused at its N-terminal end, directly or indirectly, to the C-terminal end of the following sequence
 15 SEQ ID NO 8 –

N-Met-Lys-Trp-Val-C

SEQ ID No 8

20 or a conservatively substituted variant thereof, namely -

N-Met-(Lys/Arg/His)-(Phe/Trp/Tyr)-
 (Ile/Leu/Val/Ala/Met)-C

SEQ ID No. 33

25

Additionally or alternatively it may be fused at its C-terminal end, directly or indirectly, to the N-terminal end of at least one of the following sequences –

30

N-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-C

SEQ ID No 9

or a conservatively substituted variant thereof, namely -

5 N-(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-
(Phe/Trp/Tyr)-(Ser/Thr/Gly/Tyr/Ala)-(Ser/Thr/Gly/Tyr/Ala)-
(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-(Ser/Thr/Gly/Tyr/Ala)-C

SEQ ID No. 10

or

10 N-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Ser-Leu-Asp-Lys-Arg-C

SEQ ID No 11

or

N-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Gly-Val-Phe-Arg-Arg-C

SEQ ID No. 30

15 The sequence given in SEQ ID No 9 represents the final nine amino acids of
the natural human albumin pre sequence. In the case of SEQ ID No 11, this
is fused to the final six amino acids of one of the two principal fused leader
sequences of WO 90/01063 and, in the case of SEQ ID No. 30, SEQ ID No.
9 is fused to the final six amino acids of the natural human albumin pro
20 sequence.

Preferably, in each case, X¹ is F, X² is I, X³ is V, X⁴ is as previously stated
and X⁵ is I.

25 In a preferred embodiment, the pentapeptide is fused at its N-terminal to the
C-terminal of sequence of SEQ ID NO 8 or a conservatively substituted
variant thereof and is fused at its C-terminal to the N-terminal of the
sequence of SEQ ID NO 9, a conservatively substituted variant thereof,
SEQ ID No. 10, 11 or 30, thereby to form, for example, one of the
30 following sequences -

N-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-
Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-C

SEQ ID No 12

5 or

N-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-
(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-(Ser/Thr/Gly/Tyr/Ala)-
(Ser/Thr/Gly/Tyr/Ala)-(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-
(Ser/Thr/Gly/Tyr/Ala)-C

10

SEQ ID No 13

or

N-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-
Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Ser-Leu-Asp-Lys-Arg-C

SEQ ID No 14

15

N-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-
Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Gly-Val-Phe-Arg-Arg-C

SEQ ID No 31

20 wherein X₁-X₅ are as defined above, or a conservatively substituted variant thereof, as defined above.

An especially preferred embodiment has, as the secretion pre sequence, the sequence of SEQ ID NO 28 -

25

N-Met-Lys-Trp-Val-Phe-Ile-Val-Ser-Ile-Leu-Phe-Leu-Phe-Ser-Ser-Ala-
Tyr-Ser-C

SEQ ID No 28

i.e. the pre sequence is derived from the human serum albumin secretion pre sequence, X₁, X₂, X₃, X₄ and X₅ are at positions -20, -19, -18, -17 and -16, and X₁, X₂, X₃, X₄ and X₅ are as defined by SEQ ID No.7.

5 As is apparent from above, a secretion pre sequence as defined above, such as the sequences of SEQ ID Nos 12 or 28, may be combined with secretion pro sequences to form functional pre-pro secretion sequences. In a preferred embodiment, a pre sequence motif is fused by a peptide bond at its C-terminal end to the N-terminal amino acid of a secretion pro sequence
10 motif, thereby to form a pre-pro sequence motif. It may be preferable to use a pro sequence derived from the immature version of the mature protein to which the leader sequence is, or is intended to be, attached. It may also be preferable to use the pro sequence that is associated in nature with the unmodified pre sequence or a pro sequence, or part thereof, from an related
15 leader.

Preferably, the pro sequence terminates at its C-terminus in a dibasic pair of amino acids, i.e. each is Lys or Arg.

20 Typically the secretion pro sequence motif is an albumin secretion pro sequence or variant thereof, such a variant including the dibasic pair of amino acids and having only conservative substitutions at the other positions, usually a human albumin secretion pro sequence, i.e. having the sequence N-Arg-Gly-Val-Phe-Arg-Arg-C or variant thereof. In another
25 preferred embodiment the pro sequence comprises the sequence of the whole or part of the yeast MF α -1 secretion pro sequence, i.e. N-Ser-Leu-Asp-Lys-Arg-C or variant thereof as defined for the albumin pro sequence.

In comparison with the corresponding parts of the leader defined in WO
30 90/01063 and the human albumin leader, a polypeptide of the present

invention has at least four amino acid changes namely Ser-20Phe or Trp or Tyr; Phe-19Ile or Leu or Val or Ala or Met; Ile-18Leu or Val or Ala or Met; and Leu-16Ile or Val or Ala or Met, where the notation means that, taking the first-named mutation as an example, the serine residue at position -20 (i.e. minus twenty relative to the N-terminus of the mature protein that is to be secreted using the leader sequence) is changed to a phenylalanine residue. This is exemplified in Fig. 1.

One preferred pre-pro sequence comprises the sequence:

MKWVFIVSILFLFSSAYSRY¹Y²Y³Y⁴Y⁵

wherein Y¹ is Gly or Ser, Y² is Val or Leu, Y³ is Phe or Asp, Y⁴ is Arg or Lys and Y⁵ is Arg or Lys.

In a preferred embodiment, Y¹ is Gly, Y² is Val and Y³ is Phe. In another preferred embodiment Y¹ is Ser, Y² is Leu and Y³ is Asp.

Typically Y⁴ is Arg and Y⁵ is Arg. Alternatively it is preferred if Y⁴ is Lys and Y⁵ is Arg. Another preferred alternative is where Y⁴ is Lys and Y⁵ is Lys. Y⁴ may also be Arg where Y⁵ is Lys.

An especially preferred embodiment has, as the secretion prepro sequence, the sequence of SEQ ID NO 32

N-Met-Lys-Trp-Val-Phe-Ile-Val-Ser-Ile-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Ser-Leu-Asp-Lys-Arg-C

SEQ ID No 32

A second aspect of the invention provides an isolated polynucleotide having a sequence that encodes the motif as defined by the first aspect of the invention.

5 As used herein, the term "isolated" includes the meaning that the polynucleotide, where it is a DNA molecule, is in isolation from at least most of the chromosome on which it is naturally found and, where it is an RNA molecule, is in isolation from an intact cell in which it is naturally transcribed. In other words, the polynucleotide is not claimed in a form in which it has
10 previously existed, such as in nature. Thus, a polynucleotide according to the second aspect of the invention includes a polynucleotide that has been cloned into a bacterial or fungal vector, such as a plasmid, or into a viral vector, such as a bacteriophage. Preferably such clones are in isolation from clones constituting a DNA library of the relevant chromosome.

15

The linear amino acid sequence can be reverse translated into a DNA sequence using the degenerate standard genetic code (Fig.2). in which most amino acids are encoded by more than one trinucleotide codon.

20 For example, a DNA sequence encoding the peptide defined as SEQ ID 1 would be deduced to be:

5'-(TTY/TGG/TAY)-(ATH/TTR or CTN/GTN/GCN/ATG)-(TTR or
CTN/GTN/GCN/ATG)-(NNN)-(ATH or CTN/GTN/GCN/ATG)-3'

25

SEQ ID No 15

where " 3' " and " 5' " denote the orientation of the polynucleotide sequence, rather than the actual termini; in other words, the polynucleotide sequence may be joined (e.g. fused or ligated) to other polynucleotide

sequences at either end or both ends, and wherein Y, R, H and N are as defined in Fig. 2.

Using the same conversion procedure the DNA sequence:

5

5'-TTY-ATH-GTN-(TCN or AGY)-ATH-3'

SEQ ID No 16

would be deduced to encode the polypeptide of SEQ ID No 7.

10

In the case of a polynucleotide sequence comprising a sequence that encodes a naturally occurring mature protein, such a human albumin, this can be either the naturally occurring coding sequence, such as the human albumin gene sequence, or a complementary DNA sequence (cDNA) or a
15 cDNA containing one or more introns.

Further sequence modifications may also be introduced, for example into the coding region. A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as
20 disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known
25 in the art.

The polynucleotide encoding a leader sequence of the invention is most conveniently made by chemical synthesis of an oligonucleotide, followed by ligation to the other elements of the genetic construct, by methods that
30 are well known in this art and described in more detail below.

Where it is desirable to modify the polynucleotide that encodes mature albumin, this may be most conveniently achieved by site-directed mutagenesis or PCR mutagenesis, starting from the natural cDNA sequence, or from assembling synthetic oligonucleotides. Again, such techniques are standard in this art and are in any case set out in more detail below.

Modification to the coding sequence can be advantageous because, within a particular organism, the polynucleotide sequences encoding some highly expressed proteins favour some codons over others for a particular amino acid; this is called codon bias. In a preferred embodiment of a second aspect of the invention the standard genetic code can be reduced to the preferred codons for the host organism of choice. In an especially preferred embodiment of the second aspect of the invention the standard genetic code can be reduced to the preferred codons of yeast. (See Table 4 of Sharp and Crowe (1991) *Yeast* 7, 657-678.) Advantageously this list of preferred yeast codons is modified by inclusion of the asparagine codon 5'-GAT-3' (Fig.3).

Using the peptide sequence of SEQ ID No 1 as an example, the codon biased DNA sequence encoding this peptide in yeast may be deduced to be:

5'-(TTC/TGG/TAC)-(ATY/TTG/GTY/GCT/ATG)-
(TTG/GTY/GCT/ATG)-(NNN)-(ATY/GTY/GCT/ATG)-5'

SEQ ID No 17

Using the same conversion procedure the codon-biased degenerate DNA sequence:

5'-TTC-ATY-GTY-TCY-ATY-3'

SEQ ID No 18

would be deduced for the especially preferred polypeptide motif having the sequence of SEQ ID No 7, although the most preferred codon-biased DNA sequence encoding a polypeptide motif having the sequence of SEQ ID No.

5 7 is -

TTCATCGTCTCCATT

SEQ ID No. 34

Using the genetic code given in Fig.2 or the preferred codon bias tables
10 available for the intended host or the preferred codon bias given in Fig.3, the same conversion procedure can be used to convert any desired amino acid sequence into a partially redundant polynucleotide sequence. The amino acid sequences, which can be converted into a DNA sequence by this method can be taken from, but not limited to, polypeptides according to the
15 first aspect of the invention. For example, the sequence of a coding region for mature human albumin can be derived in this way. EP 308 381 discloses a partially yeast-codon-optimised coding sequence for human albumin. SEQ ID No. 20 herein is further such sequence. Advantageously, where the DNA sequence redundancy permits, restriction sites can be
20 introduced at domain and sub-domain boundaries, without perturbing the encoded amino acid sequence (or the codon bias if Fig.3 is used).

The remaining DNA sequence redundancies can be resolved and the number of occurrences of alternative codons equalised for each amino acid
25 with redundant DNA sequences. Advantageously, DNA sequences representing possible transcription terminator sequences can be removed or reduced where possible by utilising the DNA sequence redundancy of the degenerate codons. Finally the balance of alternative codons for amino acids with redundant DNA sequences can be re-equalised but without
30 conflicting with the previous modifications

A polynucleotide according to the second aspect of the invention can be directly or indirectly fused to one or more other nucleotide sequences at its 5' and/or 3' ends, for example to form a complete gene or expression
5 cassette. Thus, the expression cassette will desirably also contain sites for transcription initiation and termination, and in the transcribed region, a ribosome binding site for translation initiation. (Hastings *et al*, WO 98/16643, published 23 April 1998.)

10 Accordingly, the second aspect of the present invention includes a polynucleotide comprising a DNA sequence that is a contiguous or non-contiguous fusion of a DNA encoding a heterologous protein with either a DNA sequence encoding a polypeptide according to the first aspect of the present invention, particularly wherein the desired protein is albumin, or a
15 variant or fragment thereof. In this context, the term "heterologous protein" means that it is not the same as the "desired protein", i.e. does not form a homodimer.

Accordingly, the polynucleotide may be directly or indirectly fused to a
20 promoter (an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur) at its 5' end and/or to other regulatory sequences, such as, at its 3' end, translation termination sequences. Thus a polynucleotide may be operably linked to one or more regulatory regions, usually transcription regulatory regions. By
25 "operably linked" is meant that the regulatory region is linked in such a way that it is able to exert an effect on the polynucleotide sequence. The choice of which regulatory region to use will be partially dependant upon the expected host (i.e. the intended expression system) and the selection of the preferred sequence will be known to those skilled in the art

Many expression systems are known, including systems employing: bacteria (eg. *Bacillus subtilis* or *Escherichia coli*) transformed with, for example, recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeasts (eg. *Saccharomyces cerevisiae* or *Pichia pastoris*) transformed with, for example, yeast expression vectors; insect cell systems transformed with, for example, viral expression vectors (eg. baculovirus); plant cell systems transfected with, for example viral or bacterial expression vectors; animal cell systems, either in cell culture, transgenic or as gene therapy, transfected with, for example, adenovirus expression vectors. The host cell is preferably a yeast (and most preferably a *Saccharomyces* species such as *S. cerevisiae* or a *Pichia* species such as *P. pastoris*).

Accordingly, a third aspect of the present invention provides a host cell transformed with a polynucleotide according to the second aspect of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells, particularly if they can secrete proteins, as can some species of *Bacillus* and *Escherichia*. Preferred eukaryotic host cells include plants, fungi, yeast and animal cells, preferably vertebrate cells, more preferably mammalian cells, such as those from a mouse, rat, cow, sheep, goat, pig, buffalo, yak, horse or other domesticated animal, monkey or human. Suitable human cells include cells from a human fibroblastic cell line. Thus a host cell may be a transgenic cell of a mammal *in situ*, and may thus be the result of a gene therapy approach or of the production of a transgenic individual. In the latter case it is preferred that the individual is a non-human mammal.

Exemplary genera of bacterial hosts include *E.coli* and *Bacillus subtilis*.

Exemplary genera of plant hosts include spermatophytes, pteridophytes (e.g. ferns, clubmosses, horsetails), bryophytes (e.g. liverworts and mosses),

and algae. Typically the plant host cell will be derived from a multicellular plant, usually a spermatophyte, such as a gymnosperm or an angiosperm. Suitable gymnosperms include conifers (e.g. pines, larches, firs, spruces and cedars), cycads, yews and ginkos. More typically the plant host cell is the
5 cell of an angiosperm, which may be a monocotyledonous or dicotyledonous plant, preferably a crop plant. Preferred monocotyledonous plants include maize, wheat, barley, sorghum, onion, oats, orchard grass and other *Pooideae*. Preferred dicotyledonous crop plants include tomato, potato, sugarbeet, cassava, cruciferous crops (including oilseed rape),
10 linseed, tobacco, sunflower, fibre crops such as cotton, and leguminous plants such as peas, beans, especially soybean, and alfalfa. The host cell may thus be an autonomous cell, for example the cell of a unicellular plant or a cell maintained in cell culture, or it may be a cell *in situ* in a multicellular plant. Accordingly the present invention contemplates the
15 production of whole transgenic plants, which preferably retain a stable and heritable transgenic phenotype.

Exemplary genera of fungal hosts include *Aspergillus* (e.g. *A. niger* and *A. oryzae*), *Streptomyces*, *Penicillium* and yeasts. Exemplary genera of yeast
20 contemplated to be useful in the practice of the present invention are *Pichia* (*Hansenula*), *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosaurus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera
25 are those selected from the group consisting of *Pichia* (*Hansenula*), *Saccharomyces*, *Kluyveromyces* and *Yarrowia*. Examples of *Saccharomyces* spp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* spp. are *K. fragilis* and *K. lactis*. Examples of *Pichia* (*Hansenula*) are *P. pastoris*, *P. anomala* and *P. capsulata*. *Y. lipolytica* is an
30 example of a suitable *Yarrowia* species. Yeast host cells include YPH499,

YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA.

5 Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

10 As discussed above, the choice of polynucleotide regulatory region will be partly dependent on the nature of the intended host.

Promoters suitable for use in bacterial host cells include the *E. coli lacI* and *lacZ* promoters, the T3 and T7 promoters, the *gpt* promoter, the phage λ PR and PL promoters, the *phoA* promoter and the *trp* promoter. Promoter
15 sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

20 Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters and the promoters of retroviral LTRs. Other suitable promoters will be known to those skilled in the art.

25 Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α -mating factor pheromone, a-
30 mating factor pheromone, the *PRB1* promoter, the *GPD1* promoter, and

hybrid promoters involving hybrids of parts of 5' regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (e.g. the promoter of EP-A-258 067).

- 5 Convenient regulatable promoters for use in *Schizosaccharomyces pombe*, another suitable host cell, are the thiamine-repressible promoter from the *nmt* gene as described by Maundrell (1990) *J. Biol. Chem.* **265**, 10857-10864 and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990) *Genetics* **124**, 807-816.

10

Suitable promoters, transformation protocols and culture conditions for *Pichia* can be found in US 5 986 062 (incorporated herein by reference). For example, preparation of an HSA-producing host (or an HSA-producing strain) may be effected using a process in which a recombinant plasmid is
15 introduced into chromosome (JP-A-3-72889 corresponding to EP-A-399455), a process in which HSA is expressed in yeast (JP-A-60-41487 corresponding to EP-A-123544, JP-A-63-39576 corresponding to EP-A-248657 and JP-A-63-74493 corresponding to EP-A-251744) and a process in which HSA is expressed in *Pichia* (JP-A-2-104290 corresponding to EP-
20 A-344459). Culturing of an HSA-producing host (an HSA production process) may be carried out using known processes, such as those referred to in US 5,986,062, for example in accordance with a process disclosed in JP-A-3-83595 or JP-A-4-293495 (corresponding to EP-A-504823). The medium for culturing a transformed host may be prepared in accordance
25 with US 5,986,062 and culturing of a host may be carried out preferably at 15 to 43°C (more preferably 20 to 30°C) for 1 to 1,000 hours, by means of static or shaking culturing or batch, semi-batch or continuous culturing under agitation and aeration in accordance with the disclosures of US 5,986,062.

30

Suitable transcription termination signals are well known in the art. Where the host cell is eukaryotic, the transcription termination signal is preferably derived from the 3' flanking sequence of a eukaryotic gene, which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, i.e. may correspond to the promoter. Alternatively, they may be different. In that case, and where the host is a yeast, preferably *S. cerevisiae*, then the termination signal of the *S. cerevisiae ADH1* gene is preferred.

10

Thus a polynucleotide according to the second aspect of the present invention can be developed for any desired host by using methods such as those described above.

15 A DNA sequence encoding mature human albumin can be developed from DNA fusions between the native gene, cDNA or a cDNA containing one or more introns, as described above and a codon biased human albumin DNA sequence derived by the method described above.

20 SEQ IQ No 19 is a polynucleotide sequence that comprises 22 nucleotides 5' to the translation initiation site, a preferred polynucleotide coding sequence for the secretion leader sequence SEQ ID No. 32 and a mature human albumin coding region SEQ ID No 20. The coding sequence ends with a translation stop codon. Typically, this is TGA, TAG or TAA, although TAA is the most efficient in yeast. Preferably, further translation stop codons (preferably each is TAA), usually one or two, are included, preferably adjacent each other or with no more than 3 base pairs between each pair of stop codons. SEQ IQ No 19 is flanked at both ends by appropriate cloning sites.

30

The polynucleotide of the second aspect of the invention may also be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion sequence(s) will depend upon the nature of the host, the manner of the introduction of the polynucleotide into the host, and whether
5 episomal maintenance or integration is desired. For example, the vectors can include a prokaryotic replicon, such as the Col E1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic cell types.

10 Generally, a polynucleotide according to the second aspect of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression.

Thus, the polynucleotide may be used in accordance with known techniques,
15 appropriately modified in view of the teachings contained herein, to construct an expression vector, including, but not limited to integration vectors, centromeric vectors and episomal vectors.

Thus in one embodiment of the second aspect of the invention, the
20 polynucleotide is a vector.

Typical prokaryotic vector plasmids are: pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories (Richmond, CA, USA); p*Trc*99A, pKK223-3, pKK233-3, pDR540 and pRIT5 available from
25 Pharmacia (Piscataway, NJ, USA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A available from Stratagene Cloning Systems (La Jolla, CA 92037, USA).

A typical mammalian cell vector plasmid is pSVL available from Pharmacia
30 (Piscataway, NJ, USA). This vector uses the SV40 late promoter to drive

expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia (Piscataway, NJ, USA). This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast episomal plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems (La Jolla, CA 92037, USA), YEp24 (Botstein, D., *et al.* (1979) *Gene* 8, 17-24), and YEplac122, YEplac195 and YEplac181 (Gietz, R.D. and Sugino. A. (1988) *Gene* 74, 527-534). Other yeast plasmids are described in WO 90/01063 and EP 424 117, as well as the "disintegration vectors of EP-A-286 424. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*, as are YIplac204, YIplac211 and YIplac128 (Gietz, R.D. and Sugino. A. (1988) *Gene* 74, 527-534). Plasmids pRS413-416 are Yeast Centromere plasmids (YCps) as are YCplac22, YCplac33 and YCplac111 (Gietz, R.D. and Sugino. A. (1988) *Gene* 74, 527-534).

20

Methods well known to those skilled in the art can be used to construct expression vectors containing the coding sequence and, for example appropriate transcriptional or translational controls. One such method involves ligation via cohesive ends. Compatible cohesive ends can be generated on the DNA fragment and vector by the action of suitable restriction enzymes. These ends will rapidly anneal through complementary base pairing and remaining nicks can be closed by the action of DNA ligase.

A further method uses synthetic double stranded oligonucleotide linkers and adaptors. DNA fragments with blunt ends are generated by bacteriophage T4

30

DNA polymerase or *E.coli* DNA polymerase I which remove protruding 3' termini and fill in recessed 3' ends. Synthetic linkers and pieces of blunt-ended double-stranded DNA which contain recognition sequences for defined restriction enzymes, can be ligated to blunt-ended DNA fragments by T4
5 DNA ligase. They are subsequently digested with appropriate restriction enzymes to create cohesive ends and ligated to an expression vector with compatible termini. Adaptors are also chemically synthesised DNA fragments which contain one blunt end used for ligation but which also possess one preformed cohesive end. Alternatively a DNA fragment or DNA fragments
10 can be ligated together by the action of DNA ligase in the presence or absence of one or more synthetic double stranded oligonucleotides optionally containing cohesive ends.

Synthetic linkers containing a variety of restriction endonuclease sites are
15 commercially available from a number of sources including Sigma-Genosys Ltd, London Road, Pampisford, Cambridge, United Kingdom.

Vectors of the invention thus produced may be used to transform an appropriate host cell for the expression and production of a polypeptide
20 comprising a sequence as defined in the first aspect of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crawl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987
25 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (2001) *Molecular Cloning, A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells. Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Physical methods may be used for introducing DNA into animal and plant cells. For example, microinjection uses a very fine pipette to inject DNA molecules directly into the nucleus of the cells to be transformed. Another example involves bombardment of the cells with high-velocity microprojectiles, usually particles of gold or tungsten that have been coated with DNA.

Plants may be transformed in a number of art-recognised ways. Those skilled in the art will appreciate that the choice of method might depend on

the type of plant targeted for transformation. Examples of suitable methods of transforming plant cells include microinjection (Crossway *et al.*, *BioTechniques* 4:320-334 (1986)), electroporation (Riggs *et al.*, *Proc. Natl. Acad. Sci. USA* 83:5602-5606 (1986), *Agrobacterium*-mediated transformation (Hinchee *et al.*, *Biotechnology* 6:915-921 (1988); see also, Ishida *et al.*, *Nature Biotechnology* 14:745-750 (1996) for maize transformation), direct gene transfer (Paszkowski *et al.*, *EMBO J.* 3:2717-2722 (1984); Hayashimoto *et al.*, *Plant Physiol.* 93:857-863 (1990) (rice)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.*, *Biotechnology* 6:923-926 (1988)). See also, Weissinger *et al.*, *Annual Rev. Genet.* 22:421-477 (1988); Sanford *et al.*, *Particulate Science and Technology* 5:27-37 (1987) (onion); Svab *et al.*, *Proc. Natl. Acad. Sci. USA* 87:8526-8530 (1990) (tobacco chloroplast); Christou *et al.*, *Plant Physiol.* 87:671-674 (1988) (soybean); McCabe *et al.*, *Bio/Technology* 6:923-926 (1988) (soybean); Klein *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:4305-4309 (1988) (maize); Klein *et al.*, *Bio/Technology* 6:559-563 (1988) (maize); Klein *et al.*, *Plant Physiol.* 91:440-444 (1988) (maize); Fromm *et al.*, *Bio/Technology* 8:833-839 (1990); and Gordon-Kamm *et al.*, *Plant Cell* 2:603-618 (1990) (maize); Koziel *et al.*, *Biotechnology* 11:194-200 (1993) (maize); Shimamoto *et al.*, *Nature* 338:274-277 (1989) (rice); Christou *et al.*, *Biotechnology* 9:957-962 (1991) (rice); Datta *et al.*, *Bio/Technology* 8:736-740 (1990) (rice); European Patent Application EP-A-332 581 (orchardgrass and other Pooideae); Vasil *et al.*, *Biotechnology* 11:1553-1558 (1993) (wheat); Weeks *et al.*, *Plant Physiol.* 102:1077-1084 (1993) (wheat); Wan *et al.*, *Plant Physiol.* 104:37-48 (1994) (barley); Jahne *et al.*, *Theor. Appl. Genet.* 89:525-533 (1994) (barley); Umbeck *et al.*, *Bio/Technology* 5:263-266 (1987) (cotton); Casas *et al.*, *Proc. Natl. Acad. Sci. USA* 90:11212-11216 (1993) (sorghum); Somers *et al.*, *Bio/Technology*

10:1589-1594 (1992) (oat); Torbert *et al.*, *Plant Cell Reports* **14**:635-640 (1995) (oat); Weeks *et al.*, *Plant Physiol.* **102**:1077-1084 (1993) (wheat); Chang *et al.*, WO 94/13822 (wheat) and Nehra *et al.*, *The Plant Journal* **5**:285-297 (1994) (wheat). *Agrobacterium*-mediated transformation is
5 generally ineffective for monocotyledonous plants for which the other methods mentioned above are preferred.

Generally, the vector will transform not all of the hosts and it will therefore be necessary to select for transformed host cells. One selection technique
10 involves incorporating into the expression vector a DNA sequence marker, with any necessary control elements, that codes for a selectable trait in the transformed cell. These markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracyclin, kanamycin or ampicillin resistance genes for culturing in *E.coli* and other bacteria.
15 Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

The marker gene can be used to identify transformants but it is desirable to determine which of the cells contain recombinant DNA molecules and which
20 contain self-ligated vector molecules. This can be achieved by using a cloning vector where insertion of a DNA fragment destroys the integrity of one of the genes present on the molecule. Recombinants can therefore be identified because of loss of function of that gene.

25 Another method of identifying successfully transformed cells involves growing the cells resulting from the introduction of an expression construct of the present invention to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.*
30 **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of

the mature protein in the supernatant of a culture of a transformed cell can be detected using antibodies.

5 In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using
10 suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal
15 culture, in a nutrient medium.

Accordingly, in a fourth aspect of the present invention there is provided a cell culture comprising a cell according to the third aspect of the invention and culture medium. Typically the culture medium will contains mature
20 polypeptide that results from the expression of a polypeptide according to the first aspect of the present invention within the expression system and, usually, by further translational processing, such as the removal of the pre and/or pro sequences.

25 Methods for culturing prokaryotic host cells, such as *E.coli*, and eukaryotic host cells, such as mammalian cells are well known in the art. Methods for culturing yeast are generally taught in EP 330 451 and EP 361 991.

Allowing host cells that have been transformed by the recombinant DNA of
30 the invention to be cultured for a sufficient time and under appropriate

conditions known to those skilled in the art in view of the teachings disclosed herein permits the expression of the polypeptide according to the first aspect of the present invention. The thus produced polypeptide may be further processed by the host cell, such that the pre and/or pro sequences are removed.
5 Accordingly the "mature" desired protein may differ from the protein as originally translated.

Thus the invention also provides, as a fifth aspect, a process for producing a mature desired protein as defined above. The process comprises the step of
10 culturing a cell according to the third aspect of the invention in a culture medium wherein the cell, as a result of the expression of a polypeptide as defined in the first aspect of the invention, secretes a mature desired protein, where it accumulates either in the periplasmic space, the culture medium or both, but preferably into the culture medium. The culture medium, which
15 contains the secreted desired protein, may then be separated from the cell(s) in the cell culture. Secreted proteins associated with the cell wall can generally be disassociated therefrom using lytic enzymes under osmotic supporting (e.g. sorbitol) conditions (which gently release the secreted protein selectively). See Elango *et al.*, *J. Biol. Chem.* **257**: 1398-1400
20 (1982). Examples of lytic enzymes suitable for this purpose include lyticase, Zymolyase-60,000, and Glusulase, all of which are commercially available, for example, the case of the latter two, from Seikagaku Kogyo or Kirin Brewery, and from Boehringer Mannheim, respectively.

25 Preferably, following the isolation of the culture medium, the mature desired protein is separated from the medium. Even more preferably the thus obtained mature desired protein is further purified.

The desired mature protein may be extracted from the culture medium by
30 many methods known in the art. For example purification techniques for

the recovery of recombinantly expressed albumin have been disclosed in: WO 92/04367, removal of matrix-derived dye; EP 464 590, removal of yeast-derived colorants; EP 319 067, alkaline precipitation and subsequent application of the albumin to a lipophilic phase; and WO 96/37515, US 5 728 553 and WO 00/44772, which describe complete purification processes; all of which are incorporated herein by reference. Proteins other than albumin may be purified from the culture medium by any technique that has been found to be useful for purifying such proteins, since the modified leader sequence of the invention will not affect the mature protein *per se*.

10

Such well-known methods include ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

The resulting protein may be used for any of its known utilities, which, in the case of albumin, include i.v. administration to patients to treat severe burns, shock and blood loss, supplementing culture media, and as an excipient in formulations of other proteins.

Although it is possible for a therapeutically useful desired protein obtained by a process of the of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers or diluents. The carrier(s) or diluent(s) must be "acceptable" in the sense of being compatible with the desired protein and not deleterious to the recipients thereof. Typically, the carriers or diluents will be water or saline which will be sterile and pyrogen free.

30

Thus, a sixth aspect of the present invention provides a process wherein a desired protein, obtained by a process according to the fifth aspect of the invention, is formulated with a therapeutically acceptable carrier or diluent thereby to produce a therapeutic product suitable for administration to a human or an animal.

The therapeutic product may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Preferred unit dosage products are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the therapeutic product may include other agents conventional in the art having regard to the type of product in question.

The invention will now be described in more detail by reference to the following non-limiting Figures and Examples wherein:

Figure 1 shows a comparison of a natural HSA leader (having pre and pro regions) (top line) with a fused HSA/MF α -1 leader sequence as disclosed in WO 90/01063 (second line) and a preferred modified leader sequence of the present invention (third line).

Figure 2 shows the standard genetic code.

Figure 3 shows a modified list of preferred *S. cerevisiae* codons.

Figure 4 shows a plasmid map of pAYE438.

Figure 5 shows a plasmid map of pAYE441.

Figure 6 shows a plasmid map of pAYE309.

Figure 7 shows a plasmid map of pAYE467.

5

Figure 8 shows a plasmid map of pAYE443.

Figure 9 shows a plasmid map of pAYE653.

10 Figure 10 shows a plasmid map of pAYE655.

Figure 11 shows a plasmid map of pAYE639.

Figure 12 shows a plasmid map of pAYE439.

15

Figure 13 shows a plasmid map of pAYE466.

Figure 14 shows a plasmid map of pAYE640.

20 Figure 15 shows plasmid maps of pAYE638 and pAYE642.

Figure 16 shows a plasmid map of pAYE643.

Figure 17 shows a plasmid map of pAYE645.

25

Figure 18 shows a plasmid map of pAYE646.

Figure 19 shows a plasmid map of pAYE647.

Figure 20 shows an analysis of rHA productivity by rocket immunoelectrophoresis. Yeast were cultured in YEP, 2% (w/v) sucrose or B/MM, 2% (w/v) sucrose for 72 hr, 200rpm at 30°C. Quantitation was performed by reference to HSA standards (mg.L⁻¹).

5

Figure 21 shows the albumin productivity in high cell density fermentation.

*Means that the human albumin level was too low to quantitate.

Figure 22 summarises the characteristics of the constructs used in the

10 examples.

Example 1

The *Saccharomyces cerevisiae* *PRB1* promoter was isolated from yeast
15 genomic DNA by PCR using two single stranded oligonucleotides PRBJM1
and PRBJM2:

PRBJM1

20 5'-GCATGCGGCCGCCCCGTAATGCGGTATCGTGAAAGCG-3'

SEQ ID NO:35

PRBJM2

5'GCATAAGCTTACCCACTTCATCTTTGCTTGTTTAG-3'

25

SEQ ID NO:36

The PCR conditions 40 cycles of 94°C for 30 seconds, 50°C for 40
seconds, 72°C for 120 seconds, followed by 72°C for 600 seconds,
followed by a 4°C hold. The 0.85kb DNA fragment was digested with both
30 *NotI* and *HindIII* and ligated into pBST+, described in WO 97/24445,
similarly digested with *NotI* and *HindIII*, to create plasmid pAYE438

(Figure 4). Plasmid pAYE438 was digested with *Hind*III and *Bam*HI and ligated with the 0.48kb *Hind*III/*Bam*HI *ADH1* terminator DNA fragment from pAYE440 previously disclosed in WO 00/44772, so as to create plasmid pAYE441 (Figure 5). Plasmid pAYE441 was linearised at the
 5 unique *Hind*III site and ligated with the 1.8kb *Hind*III/*Bsu*36I fragment from pAYE309 (Figure 6) previously disclosed (Sleep, D. *et al.* (1991) *Bio/Technology* 9, 183-187 and EP-A-0 431 880 and the double stranded oligonucleotide linker

10 5'-TTAGGCTTATA-3' SEQ ID NO: 37
 3'-CCGAATATTCGA-5' SEQ ID NO: 38

so as to create pAYE467 (Figure 7). The 3.2kb *Not*I, expression cassette from pAYE467 was ligated into *Not*I linearised pSAC35 (Sleep *et al.*
 15 (1991), *Bio/technology* 9: 183-187), which had been previously treated with calf intestinal phosphatase (CIP) to create plasmid pAYE443 (Figure 8). SEQ IQ No 22 shows a polynucleotide sequence that comprises the coding region of the HSA/MF α -1 fusion leader sequence and the mature human albumin coding region to be found within the DNA sequence of both
 20 pAYE467 and pAYE443. The polynucleotide sequence encoding the HSA/MF α -1 fusion leader sequence was modified by site directed mutagenesis with a single stranded oligonucleotide called CPK1 with the DNA sequence:

25 5'-CT AAA GAG AAA AAG AAT GGA GAC GAT GAA TAC CCA
 Ile⁻¹⁶ Val⁻¹⁸ Ile⁻¹⁹ Phe⁻²⁰
 CTT CAT CTT TGC-3' SEQ ID No 23

30

Site directed mutagenesis (SDM) was performed according to standard protocols (Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis," *Science*, **229**: 193-1210 (1985) incorporated herein by reference) although or other suitable techniques could also be used. The
5 nucleotide sequence of CPK1 was designed to modify the amino acid sequence of the HSA/MF α -1 fusion leader sequence to introduce the following mutations Thr-20Phe, Phe-19Ile, Ile-18Val and Leu-16Ile, where the numbering (-20 etc) is such that the -1 residue is the C-terminal amino acid of HSA/MF α -1 fusion leader sequence.

10

The DNA sequence of the mutagenised plasmid was confirmed by dideoxynucleotide sequencing which confirmed that the polynucleotide sequence had been mutagenised to the desired sequence and that no other DNA sequence alterations had been introduced. The new plasmid was
15 named pAYE653 (Figure 9). SEQ IQ No 24 shows a polynucleotide sequence that comprises the coding region of the modified HSA/MF α -1 fusion leader sequence and SEQ IQ No 25 shows a polynucleotide sequence that comprises the coding region of the modified HSA/MF α -1 fusion leader sequence and the mature human albumin coding region to be found within
20 the polynucleotide sequence of pAYE653.

25

The *NotI* human albumin expression cassette was isolated from pAYE653 and ligated into the unique *NotI* site of plasmid pSAC35 to generate plasmids pAYE655 (Figure 10).

Example 2

SEQ ID No 19 shows a DNA sequence that comprises: a non-coding region
30 that includes a 5' UTR from the *Saccharomyces cerevisiae* *PRB1* promoter;

a polynucleotide region encoding the modified HSA/MF α -1 fusion leader sequence of the invention; a codon optimised coding region for mature human albumin and translation termination sites.

- 5 As a control with which to compare the effects of the sequence modifications provided to the leader sequence in SEQ ID No 19, SEQ ID No 26 shows a DNA sequence that is essentially the same as SEQ ID No 19, except that, instead of the 15 polynucleotide region representing the second aspect of the invention, the DNA sequence of SEQ ID No 26
10 comprises an 15 polynucleotide region encoding the 5 amino acids of an unmodified HSA/MF α -1 fusion leader sequence, namely SFISL.

Both DNA sequences were synthesised by Genosys, Inc (Cambridge, UK) from overlapping single-stranded oligonucleotides.

15

SEQ ID No 26 was synthesised as a 1.865kb *SacI* - *HindIII* DNA fragment cloned into the *SacI* - *HindIII* sites of plasmid pBSSK- (Stratagene Europe, P.O. Box 12085, Amsterdam, The Netherlands), as plasmid pAYE639 (Figure 11).

20

The *Saccharomyces cerevisiae* *PRB1* promoter was isolated from yeast genomic DNA by PCR using two single stranded oligonucleotides PRBJM1 and PRBJM3:

25 PRBJM3

5'-GTTAGAATTAGGTTAAGCTTGTTTTTTTATTGGCGATGAA-3'

SEQ ID NO: 39

The PCR conditions 40 cycles of 94°C for 30 seconds, 50°C for 40 seconds, 72°C for 120 seconds, followed by 72°C for 600 seconds, followed by a 4°C hold. The 0.81kb DNA fragment was digested with both *NotI* and *HindIII* and ligated into pBST+, described in WO 97/24445, similarly digested with *NotI* and *HindIII*, to create plasmid pAYE439 (Figure 12). Plasmid pAYE439 was digested with *HindIII* and *BamHI* and ligated with the 0.48kb *HindIII/BamHI ADH1* terminator DNA fragment from pAYE440 previously disclosed in WO 00/44772, so as to create plasmid pAYE466 (Figure 13).

10

A 1.865kb *HindIII* DNA fragment of SEQ ID No 26 was cloned into the unique *HindIII* site of plasmid pAYE466 to create plasmid pAYE640, which was shown to contain the 1.865kb *HindIII* DNA fragment of SEQ ID No 26 between the *PRB1* promoter and the *ADH1* terminator in the correct orientation for expression from the *PRB1* promoter (Figure 14).

15

Plasmid pAYE640 was digested to completion with *NotI/PvuI* and the *NotI* 3.2kb, *PRB1* promoter/*HindIII* DNA fragment of SEQ ID No 26 gene/*ADH1* terminator expression cassette was purified. A *NotI/PvuI* double digest of pAYE640 was preferable to a single *NotI* digestion because the expression cassette (3.2kb) and pBST+ plasmid backbone (3.15kb) were similar in size. The 3.2kb *NotI*, expression cassette from pAYE640 was ligated into *NotI* linearised pSAC35 (Sleep *et al.* (1991), *Bio/technology* 9: 183-187), which had been previously treated with calf intestinal phosphatase (CIP) to create plasmid pAYE638 (Figure 15). Plasmid pAYE638 was shown to contain the *NotI* HSA expression cassette inserted into the *NotI* site of pSAC35 and orientated so that the expression of the HSA gene was away from the *LEU2* auxotrophic marker and toward the 2µm origin of replication. Plasmid pAYE642 contained the same HSA expression cassette but arranged in the opposite orientation (Figure 15).

20

25

30

SEQ ID No 19 was synthesised as a 1.865kb *SacI* - *HindIII* DNA fragment cloned into pBSSK- (Stratagene Europe, P.O. Box 12085, Amsterdam, The Netherlands), as plasmid pAYE643 (Figure 16). The DNA sequence which
5 encodes for an HSA/MF α -1 fusion leader sequence-albumin fusion within pAYE643 is given in SEQ ID No 27. The 1.865kb *HindIII* fragment of SEQ ID No 19 was isolated from pAYE643 and ligated into the unique *HindIII* site of pAYE466 to create plasmid pAYE645 (Figure 17). The *NotI* *PRB1* rHA expression cassette was isolated from pAYE645 by digestion
10 with *NotI*/*PvuI*, and ligated into the unique *NotI* site of pSAC35 to generate plasmids pAYE646 (Figure 18) and pAYE647 (Figure 19). The *NotI* expression cassette within plasmid pAYE646 was orientated in the same direction as plasmid pAYE638 and pAYE443, while the *NotI* expression cassette within plasmid pAYE647 was orientated in the opposite orientation
15 and was the same as plasmid pAYE642.

Example 3

Three different yeast strains, A, B and C, were transformed to leucine
20 prototrophy with plasmids pAYE443, pAYE638, pAYE646 and pAYE655. The transformants were patched out onto Buffered Minimal Medium (BMM, described by Kerry-Williams, S.M. *et al.* (1998) *Yeast* 14, 161-169) containing 2% (w/v) glucose (BMMD) and incubated at 30°C until grown sufficiently for further analysis. The human albumin productivity of the
25 transformants was analysed from 10mL YEP (1% (w/v) yeast extract; 2% (w/v) bacto peptone) containing 2% (w/v) glucose (YEPD) and BMMD shake flask culture (30°C, 200rpm, 72hr) by rocket immunoelectrophoresis of cell free culture supernatant (Figure 20).

The results showed that the human albumin productivity of all three strains transformed with pAYE638 was approximately 4-5 fold lower than that observed in the same strain transformed with pAYE443 (which both contained the HSA/MF α -1 fusion leader sequence, but encoded by different polynucleotide sequences) in both rich and defined media. Unexpectedly, the human albumin productivity of all three strains transformed with pAYE646 or pAYE655 was significantly higher than that observed with pAYE638 and similar or slightly greater than that observed for the same strains transformed with pAYE443.

Example 4

Yeast strain C [pAYE443], strain C [pAYE655], strain C [pAYE638] and strain C [pAYE646], and strain B [pAYE443] and strain B [pAYE646] were cultivated in high cell density fermentation in both fed-batch and fill & draw procedures, in a medium and using control parameters as described in WO 96/37515. The human albumin productivity ($Y_{P/S}$) and human albumin concentration (g/L) were assessed by scanning densitometry of SDS-PAGE of cell free whole culture. The biomass yield ($Y_{X/S}$) was also calculated from gravimetric determinations. The results (Fig. 21) indicated that, as seen previously in Example 3, the human albumin productivity ($Y_{P/S}$) and human albumin concentration (g/L) of yeast strains containing the human albumin expression plasmid pAYE638 (native polypeptide sequence but yeast-biased codons) had significantly lower productivity than the same strains containing the human albumin expression plasmid pAYE443 (native polypeptide sequence and natural codon bias for leader and mature albumin) even though the amino acid sequences of both the HSA/MF α -1 fusion leader sequence and the mature human albumin were identical.

When the strain C fermentations were run in fed-batch mode a 16% and 12% increase in human albumin productivity ($Y_{P/S}$) relative to that of pAYE443 was observed when the human albumin expression plasmids pAYE655 and pAYE646 (incorporating a modified leader sequence in accordance with the present invention) were used, respectively. When the strain B fermentations were run in fed-batch mode a 24% increase in human albumin productivity ($Y_{P/S}$) relative to that of pAYE443 was observed when the human albumin expression plasmid pAYE646 (incorporating a modified leader sequence in accordance with the present invention) was used.

When the strain C fermentations were run in fill and draw mode a 13% and 6% increase in human albumin productivity ($Y_{P/S}$) relative to that of pAYE443 was observed when the human albumin expression plasmids pAYE655 and pAYE646 (incorporating modified leader sequence in accordance with the present invention) were used, respectively. This increased to 442% and 408% relative to that of pAYE638 when the human albumin expression plasmids pAYE655 and pAYE646 (incorporating a modified leader sequence in accordance with the present invention) were used, respectively.

Summary

Plasmids pAYE443 and pAYE638 both encode human albumin having a leader sequence derived from HSA/MF α -1 fusion leader sequence, but the former uses the natural codon bias of the native polynucleotide sequences, while the latter uses a polynucleotide sequence which is fully codon optimised for yeast expression. Expression of human albumin obtained from pAYE638 is 4-5 fold lower than that obtained using pAYE443. A polynucleotide sequence encoding a modified leader sequence in accordance with the present invention has been substituted into the polynucleotide sequence encoding the HSA/MF α -1 fusion leader sequence

of both pAYE443 and pAYE638 to create the human albumin expression plasmids pAYE665 and pAYE646, respectively. The introduction of the polypeptide sequence according to the present invention led to a significant improvement in production of the desired polypeptide.

SEQ ID No.1

-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-Xaa-
(Ile/Val/Ala/Met)-

5 **SEQ ID No. 2**

-Phe-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-Xaa-(Ile/Val/Ala/Met)-

SEQ ID No. 3

-(Phe/Trp/Tyr)-Ile-(Leu/Val/Ala/Met)-Xaa-(Ile/Val/Ala/Met)-

10

SEQ ID No. 4

-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-Val-Xaa-(Ile/Val/Ala/Met)-

SEQ ID No. 5

15 -(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-Ser-
(Ile/Val/Ala/Met)-

SEQ ID No. 6

-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-Xaa-Ile-

20

SEQ ID No. 7

-Phe-Ile-Val-Ser-Ile-

SEQ ID No. 8

25 -Met-Lys-Trp-Val-

SEQ ID No. 9

-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-

SEQ ID No. 10

-(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-
(Phe/Trp/Tyr)-(Ser/Thr/Gly/Tyr/Ala)-(Ser/Thr/Gly/Tyr/Ala)-
(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-(Ser/Thr/Gly/Tyr/Ala)-

5

SEQ ID No. 11

-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Ser-Leu-Asp-Lys-Arg-

SEQ ID No. 12

10 -Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-

SEQ ID No. 13

-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-
(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-(Ser/Thr/Gly/Tyr/Ala)-
15 (Ser/Thr/Gly/Tyr/Ala)-(Ile/Leu/Val/Ala/Met)-(Phe/Trp/Tyr)-
(Ser/Thr/Gly/Tyr/Ala)-

SEQ ID No. 14

-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-
20 Arg-Ser-Leu-Asp-Lys-Arg-

SEQ ID No. 15

-(TTY/TGG/TAY)-(ATH/TTR or CTN/GTN/GCN/ATG)-(TTR or
CTN/GTN/GCN/ATG)-(NNN)-(ATH or CTN/GTN/GCN/ATG)-
25

SEQ ID No. 16

-TTY-ATH-GTN-(TCN or AGY)-ATH-

SEQ ID No. 17

-(TTC/TGG/TAC)-(ATY/TTG/GTY/GCT/ATG)-(TTG/GTY/GCT/ATG)-
(NNN)-(ATY/GTY/GCT/ATG)-

5 **SEQ ID No. 18**

-TTC-ATY-GTY-TCY-ATY-

SEQ ID NO 19:

10 AAGCTTAACCTAATTCTAACAAGCAAAGATGAAGTGGGTTTTCA
TCGTCTCCATTTTGTTCCTTGTCTCCTCTGCTTACTCTAGATCTTTG
GATAAGAGAGACGCTCACAAGTCCGAAGTCGCTCACAGATTCAA
GGACTTGGGTGAAGAAAACCTCAAGGCTTTGGTCTTGATCGCTTT
CGCTCAATACTTGCAACAATGTCCATTCGAAGATCACGTCAAGTT
15 GGTCAACGAAGTTACCGAATTCGCTAAGACTTGTGTTGCTGACG
AATCTGCTGAAAACCTGTGACAAGTCCTTGACACACCTTGTTCCGGTG
ATAAGTTGTGTACTGTTGCTACCTTGAGAGAAACCTACGGTGAA
ATGGCTGACTGTTGTGCTAAGCAAGAACCAGAAAGAAACGAATG
TTTCTTGCAACACAAGGACGACAACCCAACTTGCCAAGATTGG
20 TTAGACCAGAAGTTGACGTCATGTGTACTGCTTTCCACGACAACG
AAGAAACCTTCTTGAAGAAGTACTTGTACGAAATTGCTAGAAGA
CACCCATACTTCTACGCTCCAGAATTGTTGTTCTTCGCTAAGAGA
TACAAGGCTGCTTTCACCGAATGTTGTCAAGCTGCTGATAAGGCT
GCTTGTTTGTGTTGCCAAAGTTGGATGAATTGAGAGACGAAGGTAA
25 GGCTTCTTCCGCTAAGCAAAGATTGAAGTGTGCTTCCTTGCAAAA
GTTCCGGTGAAAGAGCTTTCAAGGCTTGGGCTGTCGCTAGATTGTC
TCAAAGATTCCCAAAGGCTGAATTCGCTGAAGTTTCTAAGTTGGT
TACTGACTTGACTAAGGTTTCACTGAATGTTGTCACGGTGACTT
GTTGGAATGTGCTGATGACAGAGCTGACTTGGCTAAGTACATCT
30 GTGAAAACCAAGACTCTATCTCTTCCAAGTTGAAGGAATGTTGTG

AAAAGCCATTGTTGGAAAAGTCTCACTGTATTGCTGAAGTTGAA
 AACGATGAAATGCCAGCTGACTTGCCATCTTTGGCTGCTGACTTC
 GTTGAATCTAAGGACGTTTGTAAAGAACTACGCTGAAGCTAAGGA
 CGTCTTCTTGGGTATGTTCTTGTACGAATACGCTAGAAGACACCC
 5 AGACTACTCCGTTGTCTTGTGTTGAGATTGGCTAAGACCTACGA
 AACTACCTTGGAAAAGTGTTGTGCTGCTGCTGACCCACACGAAT
 GTTACGCTAAGGTTTTTCGATGAATTCAAGCCATTGGTCGAAGAAC
 CACAAAACCTTGATCAAGCAAACTGTGAATTGTTTCGAACAATTG
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 10 GAAGGTCCCACAAGTCTCCACCCCAACTTTGGTTGAAGTCTCTAG
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 ACCAATTGTGTGTTTTTGCACGAAAAGACCCCAGTCTCTGATAGAG
 TCACCAAGTGTTGTACTGAATCTTTGGTTAACAGAAGACCATGTT
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 ACGCTGAAACTTTACCTTCCACGCTGATATCTGTACCTTGTCCG
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SEQ ID NO 20:

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5 GCTAGAAGACACCCATACTTCTACGCTCCAGAATTGTTGTTCTTC
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GATAAGGCTGCTTGTTTGTGTTGCCAAAGTTGGATGAATTGAGAGA
CGAAGGTAAGGCTTCTTCCGCTAAGCAAAGATTGAAGTGTGCTT
CCTTGCAAAAGTTCGGTGAAAGAGCTTTC AAGGCTTGGGCTGTC
10 GCTAGATTGTCTCAAAGATTCCCAAAGGCTGAATTCGCTGAAGTT
TCTAAGTTGGTTACTGACTTGACTAAGGTTCACTGAATGTTGT
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15 GAAGTTGAAAACGATGAAATGCCAGCTGACTTGCCATCTTTGGC
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AGCTAAGGACGTCTTCTTGGGTATGTTCTTGTACGAATACGCTAG
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AAGGCTGATGATAAGGAACTTGTTCGCTGAAGAAGGTAAGAA
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5

SEQ ID NO 21:

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GAAGATCACGTCAAGTTGGTCAACGAAGTTACCGAATTCGCTAA
GACTTGTGTTGCTGACGAATCTGCTGAAAAGTGTGACAAGTCCTT
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30 TTTGGCTGCTGACTTCGTTGAATCTAAGGACGTTTGTAAGAACTA

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20 **SEQ ID NO 22:**

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25 CTTGGTGTTGATTGCCTTTGCTCAGTATCTTCAGCAGTGTCCATTT
GAAGATCATGTAAAATTAGTGAATGAAGTAACTGAATTTGCAAA
AACATGTGTTGCTGATGAGTCAGCTGAAAATTGTGACAAATCAC
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GTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAA
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 10 GGAATGCTGCCATGGAGATCTGCTTGAATGTGCTGATGACAGGG
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5 **SEQ ID NO 23**

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TGC

SEQ ID NO 24

10

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SEQ ID NO 25

15

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SEQ ID NO 26

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TAAGAAGTTGGTCGCTGCTTCCCAAGCTGCTTTGGGTTTG

SEQ ID NO 27

15 ATGAAGTGGGTTTTTCATCGTCTCCATTTTGTCTTGTTCTCCTCTG
CTTACTCTAGATCTTTGGATAAGAGA

SEQ ID NO 28

20 N-Met-Lys-Trp-Val-Phe-Ile-Val-Ser-Ile-Leu-Phe-Leu-Phe-Ser-Ser-Ala-
Tyr-Ser-C

SEQ ID No 29

25 N-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-(Leu/Val/Ala/Met)-Thr-
(Ile/Val/Ala/Met)-C

SEQ ID No 30

30 N-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-Ser-Arg-Gly-Val-Phe-Arg-Arg-C

SEQ ID No 31

5 N-Met-Lys-Trp-Val-X₁-X₂-X₃-X₄-X₅-Leu-Phe-Leu-Phe-Ser-Ser-Ala-Tyr-
Ser-Arg-Gly-Val-Phe-Arg-Arg-C

SEQ ID No 32

10 N-Met-Lys-Trp-Val-Phe-Ile-Val-Ser-Ile-Leu-Phe-Leu-Phe-Ser-Ser-Ala-
Tyr-Ser-Arg-Ser-Leu-Asp-Lys-Arg-C

SEQ ID No. 33

15 -Met-(Lys/Arg/His)-(Phe/Trp/Tyr)-(Ile/Leu/Val/Ala/Met)-

SEQ ID No. 34

-TTCATCGTCTCCATT-

20

SEQ ID No. 35

5'-GCATGCGGCCGCCCCGTAATGCGGTATCGTGAAAGCG-3'

SEQ ID No. 36

25 5'-GCATAAGCTTACCCACTTCATCTTTGCTTGTTTAG-3'

SEQ ID No. 37

5'-TTAGGCTTATA-3'

SEQ ID No.38

5'-AGCTTATAAGCC-3'

SEQ ID No.39

5 5'-GTTAGAATTAGGTTAAGCTTGTTTTTTTATTGGCGATGAA-3'

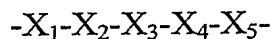
CLAIMS

1. A polypeptide comprising

(i) a leader sequence, the leader sequence comprising

5 (a) a secretion pre sequence, and

(b) the following motif:



10 where X_1 is phenylalanine, tryptophan, or tyrosine, X_2 is isoleucine, leucine, valine, alanine or methionine, X_3 is leucine, valine, alanine or methionine, X_4 is any amino acid and X_5 is isoleucine, valine, alanine or methionine; and

(ii) a desired protein heterologous to the leader sequence.

15

2. A polypeptide according to Claim 1 wherein X_1 is phenylalanine.

3. A polypeptide according to Claim 1 or 2 wherein X_2 is isoleucine.

20 4. A polypeptide according to any one of the preceding claims wherein X_3 is valine.

5. A polypeptide according to any one of the preceding claims wherein X_4 is serine or threonine, glycine, alanine or methionine.

25

6. A polypeptide according to any one of the preceding claims wherein X_4 is serine or threonine.

7. A polypeptide according to any one of the preceding claims wherein the amino acids of the motif are included in the polypeptide as substitutes, for naturally occurring amino acids.
- 5 8. A polypeptide according to Claim 7 wherein X_4 is the naturally occurring amino acid at that position, or a variant thereof.
9. A polypeptide according to any one of the preceding claims wherein X_5 is isoleucine.
- 10 10. A polypeptide according to any one of the preceding claims wherein the motif is -Phe-Ile-Val-Ser-Ile-.
11. A polypeptide according to any one of the preceding claims wherein
15 the secretion pre sequence is an albumin secretion pre sequence or a variant thereof.
12. A polypeptide according to Claim 11 wherein X_1 , X_2 , X_3 , X_4 and X_5 are at positions -20, -19, -18, -17 and -16, respectively, in place of the
20 naturally occurring amino acids at those positions, wherein the numbering is such that the -1 residue is the C-terminal amino acid of the native albumin secretion pro sequence and where X_1 , X_2 , X_3 , X_4 and X_5 are amino acids as defined in any one of Claims 1 to 10.
- 25 13. A polypeptide according to Claim 11 or 12 wherein the albumin secretion pre sequence or variant thereof is a human albumin secretion pre sequence or a variant thereof.
14. A polypeptide according to Claim 13 comprising the secretion pre
30 sequence MKWVFIVSILFLFSSAYS.

15. A polypeptide according to any one of the preceding claims wherein the leader sequence comprises a secretion pro sequence.

5 16. A polypeptide according to Claim 15 wherein the albumin secretion pre sequence or variant thereof is fused by a peptide bond at its C-terminal end to the N-terminal amino acid of a secretion pro sequence, or variant thereof, thereby to form a pre-pro sequence.

10 17. A polypeptide according to Claim 15 or 16 wherein the secretion pro sequence is an albumin secretion pro sequence or variant thereof.

15 18. A polypeptide according to Claim 17 wherein the albumin secretion pro sequence is human serum albumin secretion pro sequence or variant thereof.

19. A polypeptide according to Claim 15 or 16 wherein the secretion pro sequence motif is the yeast MF α -1 secretion pro sequence or variant thereof.

20 20. A polypeptide according to Claim 15 comprising the sequence:

MKWVFIVSILFLFSSAYSRY¹Y²Y³Y⁴Y⁵

25 wherein Y¹ is Gly or Ser, Y² is Val or Leu, Y³ is Phe or Asp, Y⁴ is Arg or Lys and Y⁵ is Arg or Lys, or variants thereof.

21. A polypeptide according to Claim 20 wherein Y¹ is Gly, Y² is Val and Y³ is Phe; or Y¹ is Ser, Y² is Leu and Y³ is Asp.

30

22. A polypeptide according to Claim 20 or 21 wherein Y⁴ is Arg and Y⁵ is Arg; Y⁴ is Lys and Y⁵ is Arg; Y⁴ is Lys and Y is Lys; or Y⁴ is Arg and Y⁵ is Lys.
- 5 23. A polypeptide according to any one of claims 1 to 10 wherein at least part of said motif is present in the secretion pre-sequence.
24. A polypeptide according to any one of the preceding claims wherein the sequence of the desired protein is fused at its N-terminal end to the C-
10 terminal amino acid of the leader sequence.
25. A polypeptide according to any one of the preceding claims where the desired protein is albumin or a variant, fragment or fusion thereof.
- 15 26. A polypeptide according to Claim 25 wherein the albumin is human albumin.
27. A polypeptide according to any one of Claims 1 to 24 wherein the mature polypeptide is transferrin or a variant, fragment or fusion thereof.
- 20 28. A polypeptide according to Claim 27 wherein the transferrin is human transferrin.
29. An isolated polynucleotide comprising a sequence that encodes the
25 motif defined by any preceding claim.
30. A polynucleotide according to Claim 29 comprising the sequence of SEQ ID No. 15.

31. A polynucleotide according to Claim 29 comprising the sequence of
SEQ ID No. 16.
32. A polynucleotide according to Claim 29 comprising the sequence of
5 SEQ ID No. 17.
33. A polynucleotide according to Claim 29 comprising the sequence of
SEQ ID No. 18.
- 10 34. A polynucleotide according to Claim 29 comprising the sequence of
SEQ ID No. 34.
35. A polynucleotide according to Claim 33 or 34 comprising the
sequence of SEQ ID No. 24.
- 15 36. A polynucleotide according to Claim 35 comprising the sequence of
SEQ ID No. 25 or a variant thereof, which variant has the leader sequence
of SEQ ID No.24 and encodes a variant or fragment of the albumin encoded
by SEQ ID No.25.
- 20 37. A polynucleotide according to Claim 33 or 34 comprising the
sequence of SEQ ID No. 27.
38. A polynucleotide according to Claim 37 comprising the sequence of
25 SEQ ID No. 21 or a variant thereof, which variant has the leader sequence
of SEQ ID No.27 and encodes a variant or fragment of the albumin encoded
by SEQ ID No.21.
39. A polynucleotide comprising the sequence of SEQ ID No. 21 or
30 fragment thereof.

40. A polynucleotide according to any one of Claims 36, 38 or 39 wherein the polynucleotide comprises a DNA sequence being a contiguous or non-contiguous fusion of a DNA sequence encoding a heterologous protein with either the DNA sequence SEQ ID No. 25 or the DNA sequence SEQ ID No. 21.

41. A polynucleotide which is the complementary strand of a polynucleotide according to any one of claims 29 to 40.

10

42. A polynucleotide according to any one of Claims 29 to 41 comprising an operably linked transcription regulatory region.

43. A polynucleotide according to Claim 42 wherein the transcription regulatory region comprises a transcription promoter.

15

44. A self-replicable polynucleotide sequence comprising a polynucleotide according any one of Claims 29 to 43.

45. A cell comprising a polynucleotide according to any one of Claims 29 to 44.

20

46. A cell according to Claim 45 which is a eukaryotic cell.

47. A cell according to Claim 46 which is a fungal cell.

25

48. A cell according to Claim 47 which is an *Aspergillus* cell

49. A cell according to Claim 47 which is a yeast cell.

30

50. A cell according to Claim 49 which is a *Saccharomyces*, *Kluyveromyces*, *Schizosaccharomyces* or *Pichia* cell.

51. A cell culture comprising a cell according to any one of Claims 45 to
5 50 and culture medium.

52. A cell culture according to Claim 51 wherein the medium contains a mature desired protein as a result of the production of a polypeptide as defined in any one of Claims 1 to 25.

10

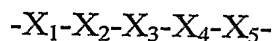
53. A process for producing a mature desired protein, comprising (1) culturing a cell according to any one of Claims 45 to 50 in a culture medium wherein the cell, as a result of the production of a polypeptide as defined in any one of Claims 1 to 28, secretes a mature desired protein into the culture
15 medium, and (2) separating the culture medium, containing the secreted mature protein, from the cell.

54. A process according to Claim 53 additionally comprising the step of separating the mature desired protein from the medium and optionally
20 further purifying the mature desired protein.

55. A process according to Claim 54 additionally comprising the step of formulating the thus separated and/or purified mature desired protein with a therapeutically acceptable carrier or diluent thereby to produce a therapeutic
25 product suitable for administration to a human or an animal.

ABSTRACT
GENE AND POLYPEPTIDE SEQUENCES

- 5 The present invention provides a polypeptide comprising (i) a leader sequence, the leader sequence comprising a (a) secretion pre sequence, and (b) the following motif:



10

- where X_1 is phenylalanine, tryptophan, or tyrosine, X_2 is isoleucine, leucine, valine, alanine or methionine, X_3 is leucine, valine, alanine or methionine, X_4 is any amino acid and X_5 is isoleucine, valine, alanine or methionine; and (ii) a desired protein heterologous to the leader sequence. A
- 15 polypeptide of the invention may additionally comprise, as part of the leader sequence, a secretion pro sequence. The invention also provides a polynucleotide comprising a sequence that encodes a polypeptide of the invention and a cell, preferably a yeast cell, comprising said polynucleotide.

Figure 1

Fig.1

5

<u>Pre</u>	<u>Pro</u>	-----pre-albumin----- -----pro region-----
HSA	HSA	Met Lys Trp Val Thr Phe Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ser Ala Tyr Ser Arg Gly Val Phe Arg Arg
HSA	MF α -1	Met Lys Trp Val Thr Phe Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ser Ala Tyr Ser Arg Ser Arg Ser Leu Asp Lys Arg
		-24 -23 -22 -21 -20 -19 -18 -17 -16 -15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1
	Preferred mutations	
	of the invention:	Phe Ile Val Ile

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Fig.2**Standard genetic code**

	T	C	A	G
T	TTT Phe (F) TTC Phe (F) TTA Leu (L) TTG Leu (L)	TCT Ser (S) TCC Ser (S) TCA Ser (S) TCG Ser (S)	TAT Tyr (Y) TAC Tyr (Y) TAA Ter TAG Ter	TGT Cys (C) TGC Cys (C) TGA Ter TGG Trp (W)
C	CTT Leu (L) CTC Leu (L) CTA Leu (L) CTG Leu (L)	CCT Pro (P) CCC Pro (P) CCA Pro (P) CCG Pro (P)	CAT His (H) CAC His (H) CAA Gln (Q) CAG Gln (Q)	CGT Arg (R) CGC Arg (R) CGA Arg (R) CGG Arg (R)
A	ATT Ile (I) ATC Ile (I) ATA Ile (I) ATG Met (M)	ACT Thr (T) ACC Thr (T) ACA Thr (T) ACG Thr (T)	AAT Asn (N) AAC Asn (N) AAA Lys (K) AAG Lys (K)	AGT Ser (S) AGC Ser (S) AGA Arg (R) AGG Arg (R)
G	GTT Val (V) GTC Val (V) GTA Val (V) GTG Val (V)	GCT Ala (A) GCC Ala (A) GCA Ala (A) GCG Ala (A)	GAT Asp (D) GAC Asp (D) GAA Glu (E) GAG Glu (E)	GGT Gly (G) GGC Gly (G) GGA Gly (G) GGG Gly (G)

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Single letter code:

A = adenosine

C = cytidine

G = guanosine

10 T = thymidine

B = C or G or T

D = A or G or T

H = A or C or T

K = G or T

15 M = A or C

N = A or C or G or T

R = A or G

S = C or G

V = A or C or G

20 W = A or T

Y = C or T

Fig.3**Modified list of preferred yeast codons**

	T	C	A	G
T	TTC Phe (F) TTG Leu (L)	TCT Ser (S) TCC Ser (S)	TAC Tyr (Y) TAA Ter	TGT Cys (C) TGG Trp (W)
C		CCA Pro (P)	CAT His (H) CAA Gln (Q)	
A	ATT Ile (I) ATC Ile (I) ATG Met (M)	ACT Thr (T) ACC Thr (T)	AAC Asn (N) AAG Lys (K)	AGA Arg (R)
G	GTT Val (V) GTC Val (V)	GCT Ala (A)	GAT Asp (D) GAC Asp (D) GAA Glu (E)	GGT Gly (G)



Fig.4

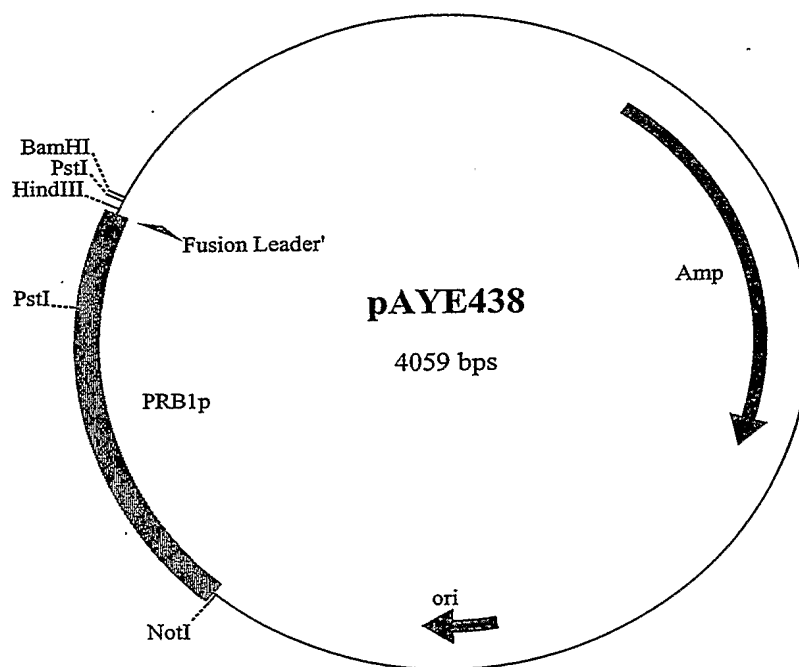




Fig.5

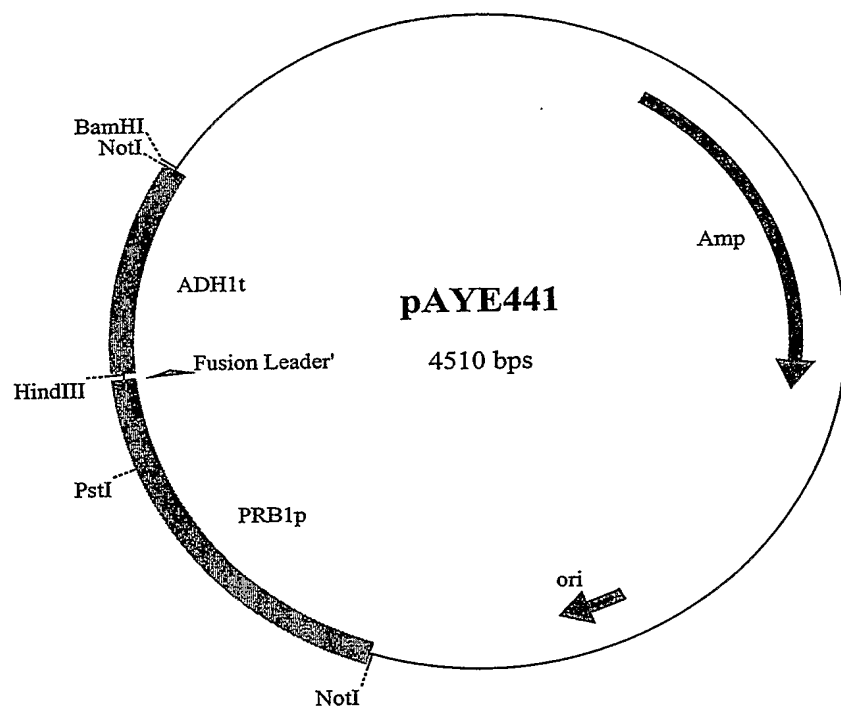




Fig. 6

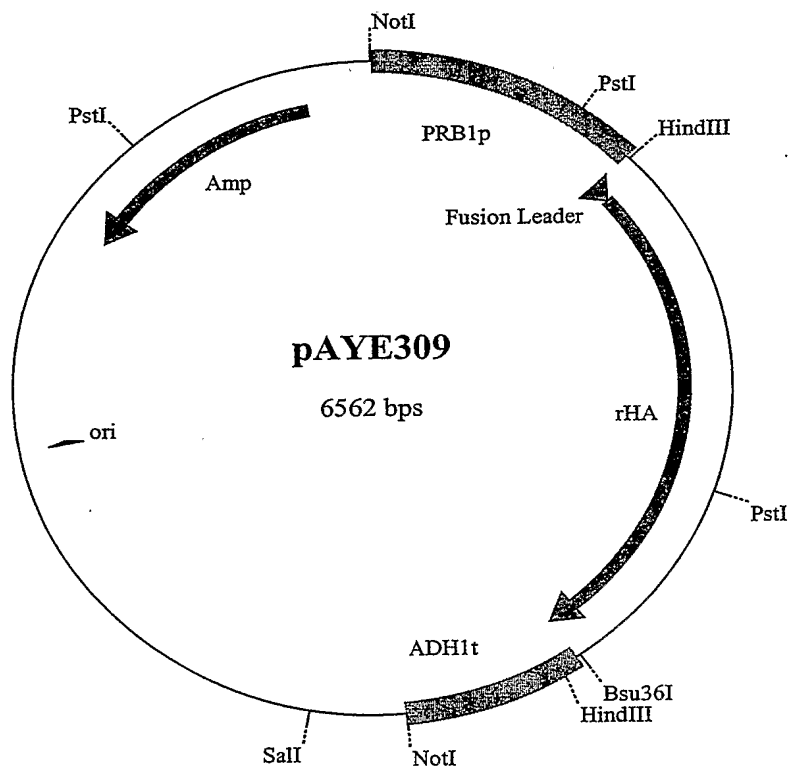




Fig. 7

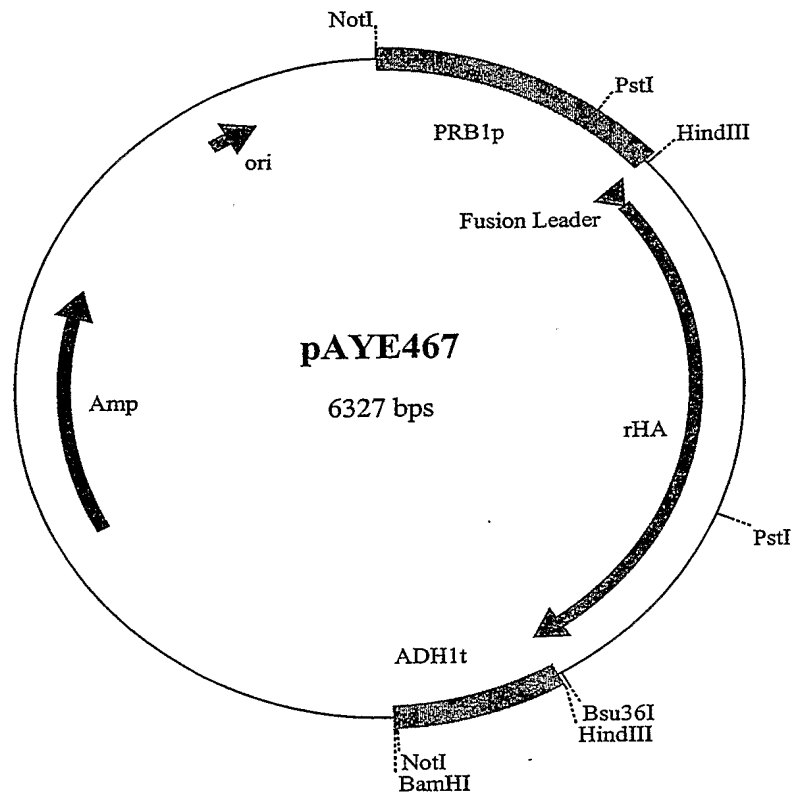




Fig.8

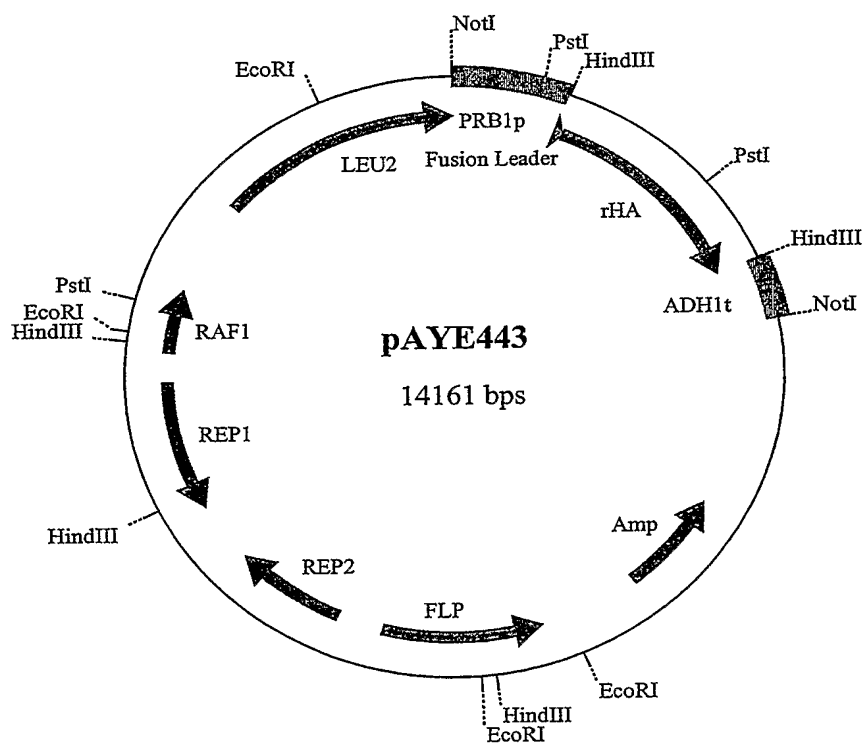




Fig.9

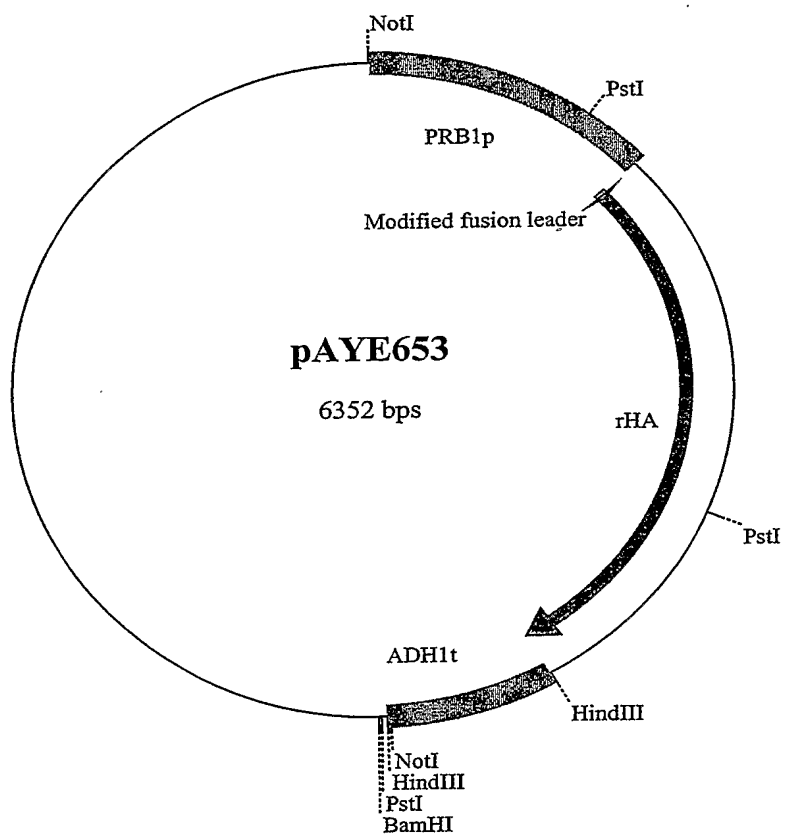




Fig.10

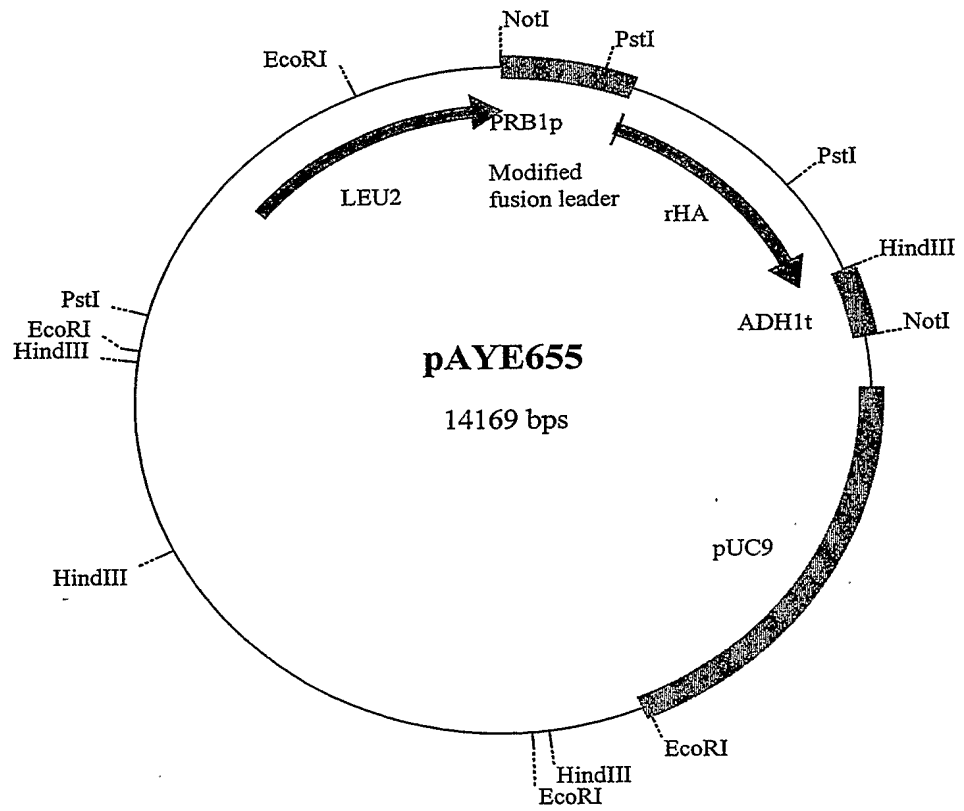




Fig.11

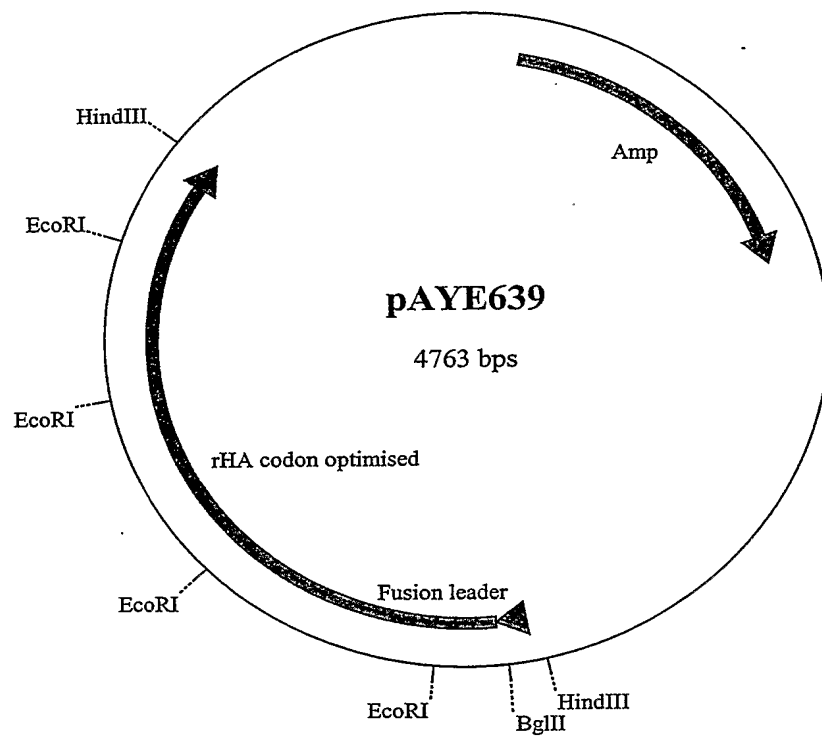




Fig.12

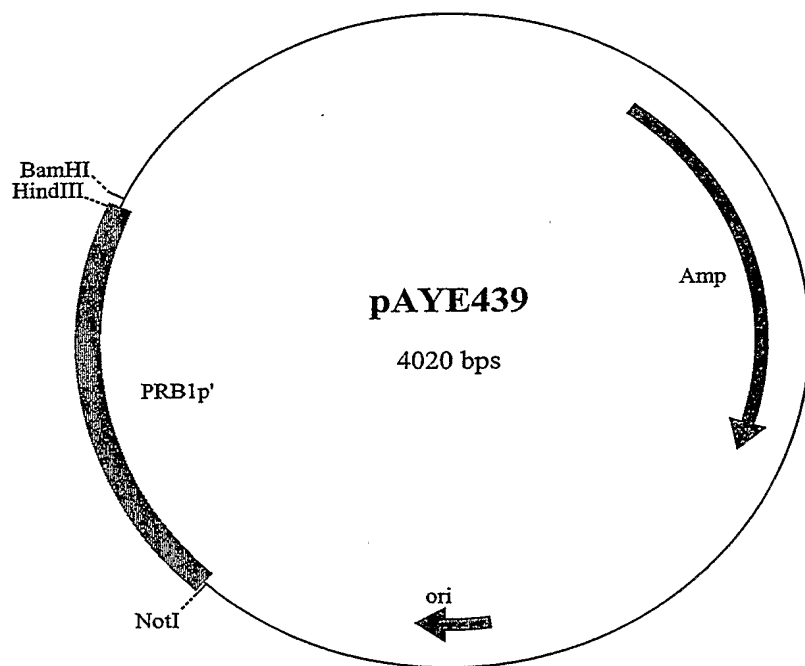




Fig.13

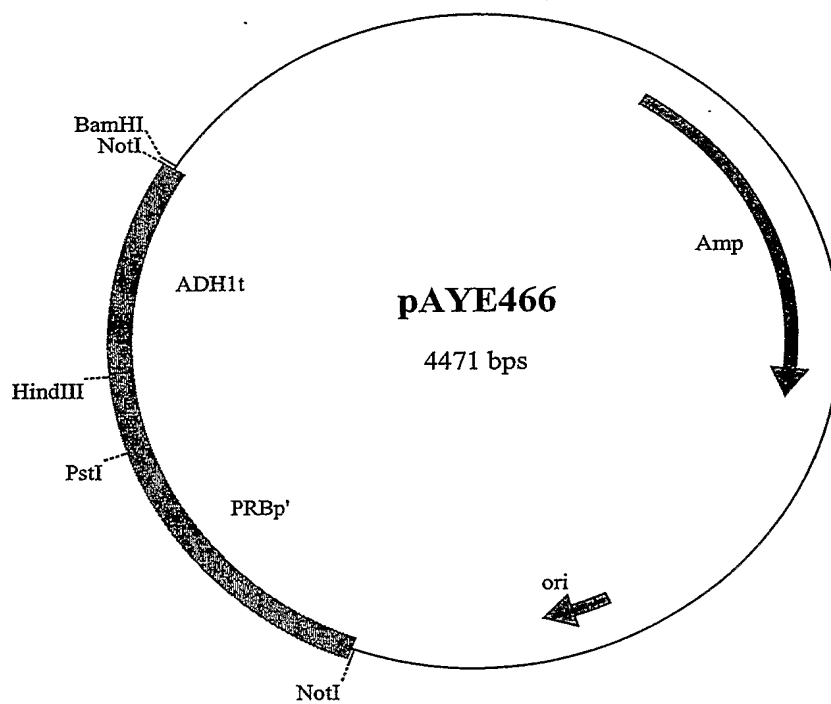




Fig.14

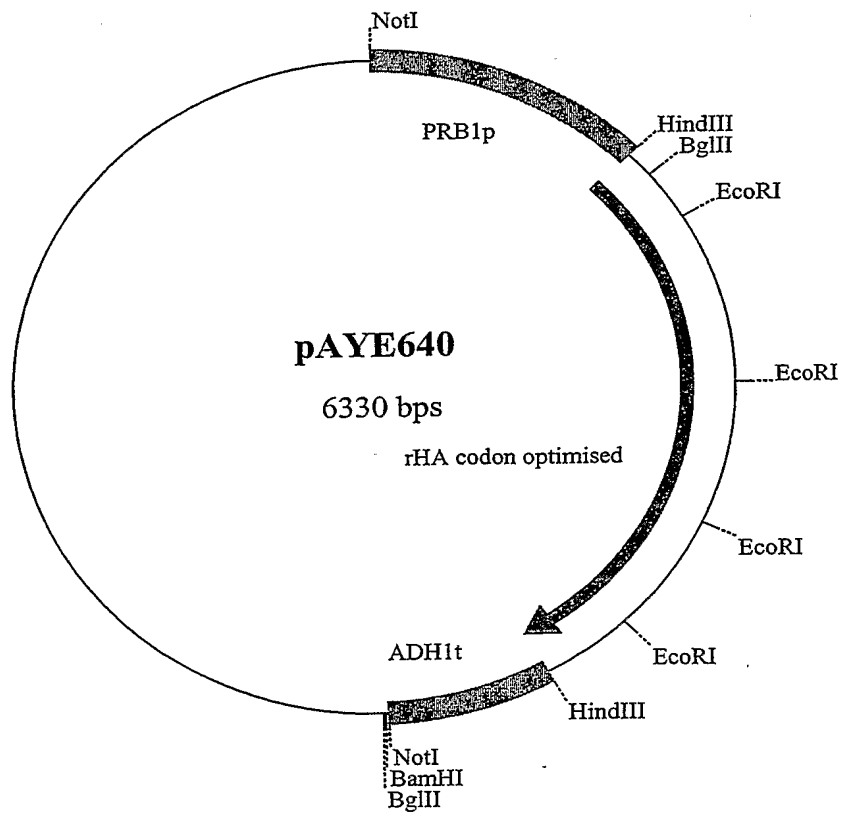




Fig.15

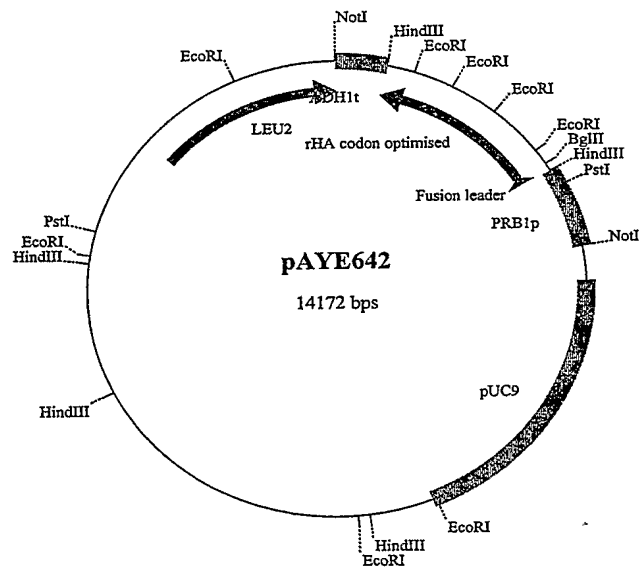
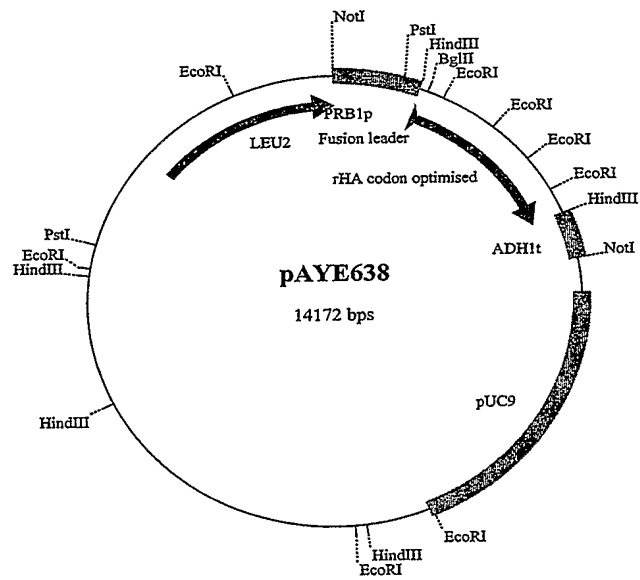




Fig.16

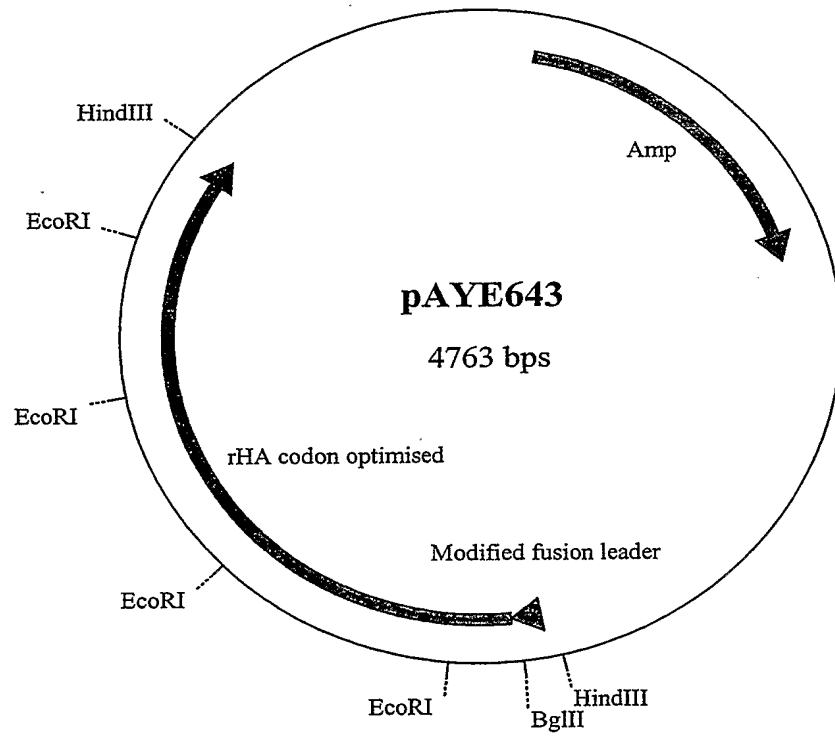




Fig.17

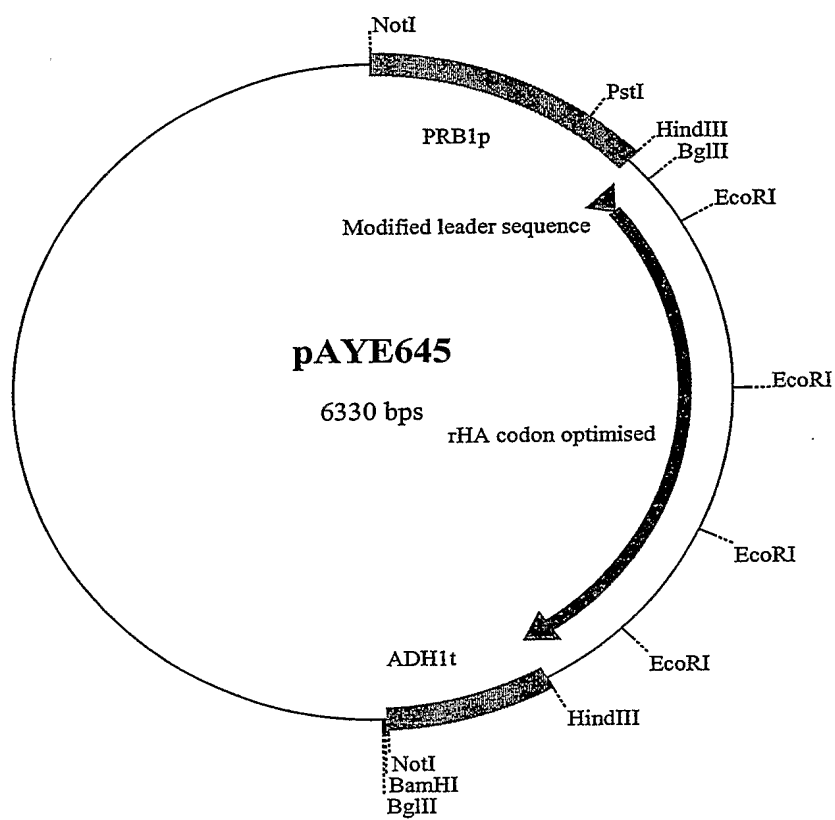




Fig.18

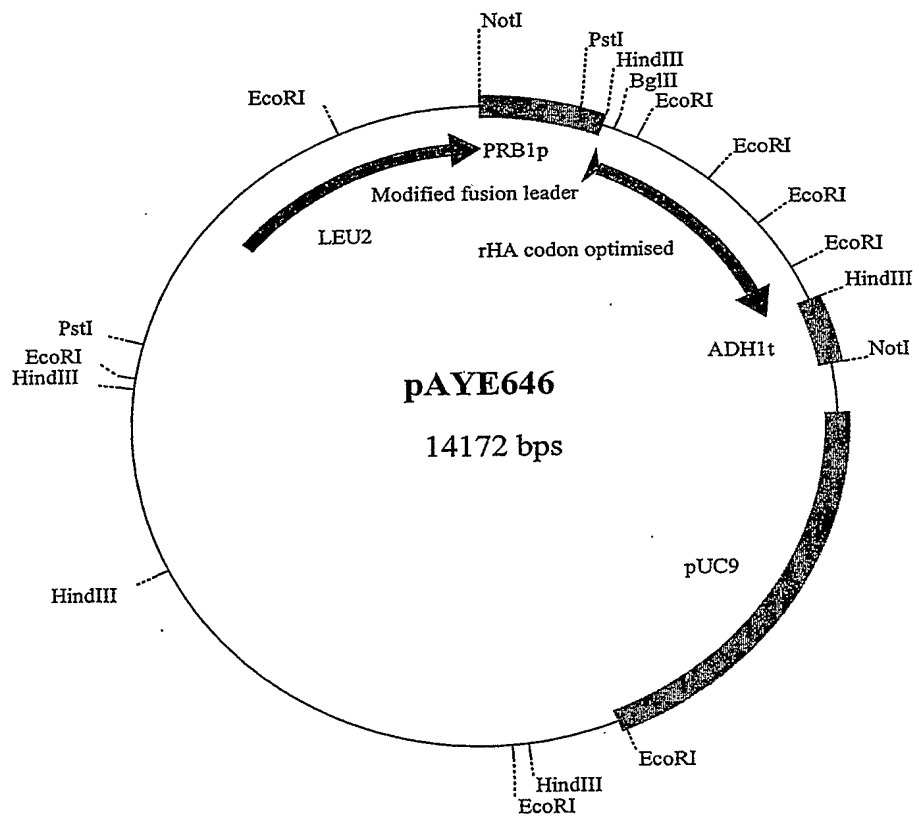


Fig.19

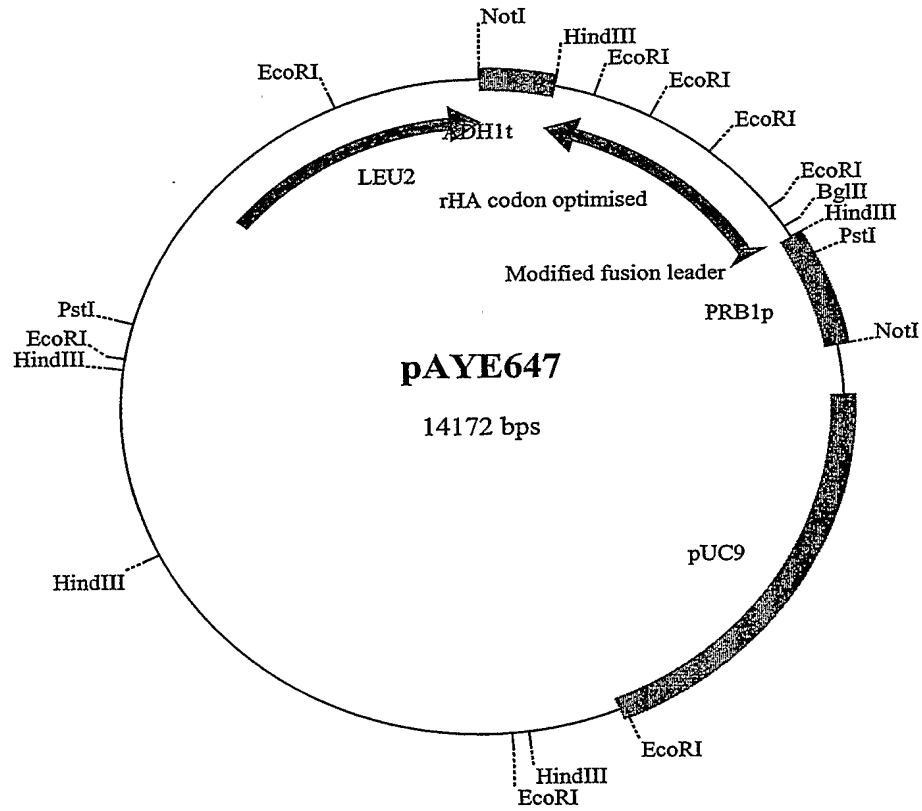


Fig. 20

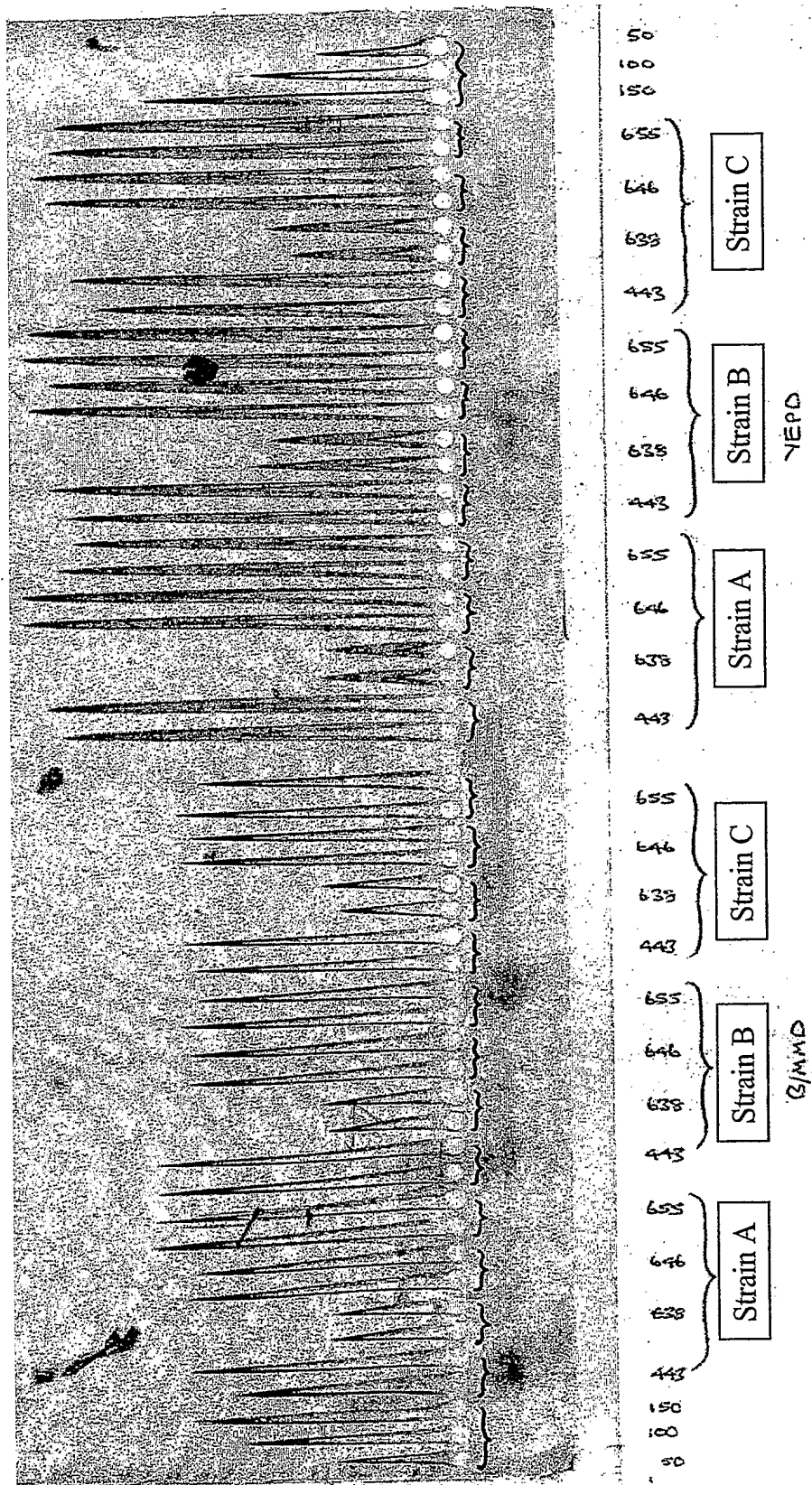




Fig.21

Strain	Plasmid	Yx/s (g/g)	Yp/s (mg/g)	rHA (g/L)
C	pAYE443 (1 st feeds)	0.33	10.4	2.9
	(fill and draw)	0.34	11.5	3.2
	pAYE638 (1 st feeds)	0.36	*	*
	(fill and draw)	0.36	2.4	0.7
	pAYE646 (1 st feeds)	0.33	11.6	3.2
	(fill and draw)	0.35	12.2	3.5
	pAYE655 (1 st feeds)	0.37	12.1	3.4
	(fill and draw)	0.35	13.0	3.7
B	pAYE443 (1 st feeds)	0.35	10.5	2.8
	pAYE646 (1 st feeds)	0.35	13.0	3.5
	(fill and draw)	0.33	12.8	3.6



Fig.22

Plasmid designation	Leader	Desired Protein
pAYE443 pAYE467	HSA/MF α -1 natural codon bias	HSA natural codon bias
pAYE655 pAYE643	modified HSA/MF α -1 FIVSI fully codon biased the rest has natural codon bias	HSA natural codon bias
pAYE638 pAYE639 pAYE640 pAYE642	HSA/MF α -1 all fully codon biased	HSA fully codon biased
pAYE645 pAYE646 pAYE647	modified HSA/MF α -1 all fully codon biased	HSA fully codon biased

THE PATENT OFFICE
05 SEP 2003
Received in Patents
International Unit